Cellular and molecular biomarkers detected in the oral mucosa and saliva in water-pipe tobacco smoking compared to cigarette smoking: A systematic review

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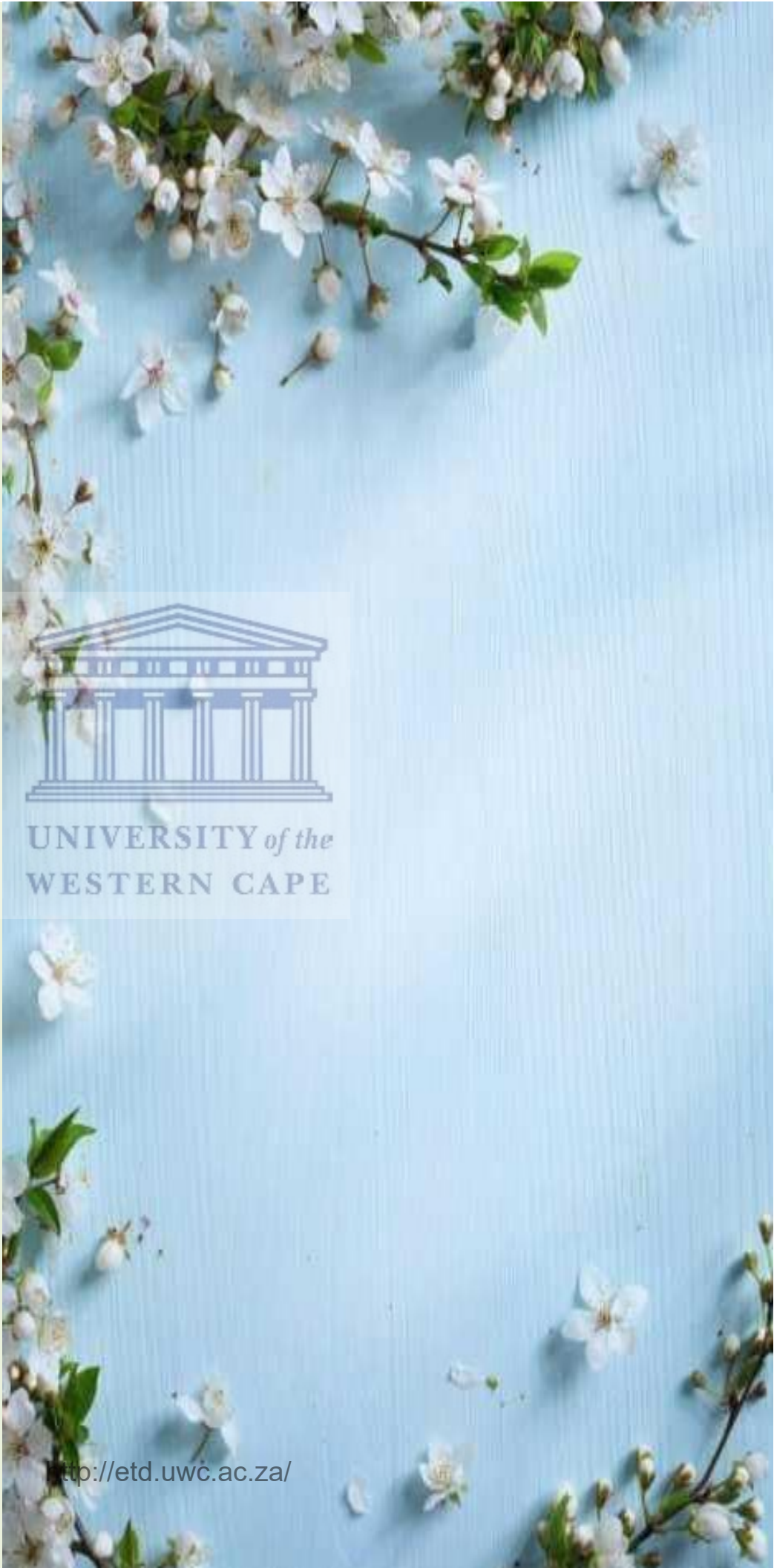

Dedication

This Research is passionately dedicated to my adorable kids (Kamaleldin , Ahmed and Zain), who have been always the source of my strength .To my loving parents (Ahmed and Salma) who were always believed on me, your wards of encouragements were the reason of my success through my life .To my amazing husband (Khalid) for all your support, you gave me strength when I thought of giving up .To my brothers, sisters , my supervisors (Prof Tina and Dr. Faheema) and my friends (Salma , Mai and Muzan) who shared their advice and assistance to complete this research .And ,finally I would like to thanks god for guidance ,skills and protection.

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Cellular and molecular biomarkers detected in the oral mucosa and saliva in water-pipe tobacco smoking compared to cigarette smoking:

A systematic review

Master's Thesis

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TABLE OF CONTENT

Abbreviations

Basal cells (BC)	**Jordanian Dinar (JD)**
Binucleated cells (BN)	**Mean Number (mn)**
Broken eggs (BE)	**Micronuclei (MN)**
Carbon Monoxide (C.O.)	**nuclear-cytoplasmic ratio (N/C)**
Cigarettes smokers (CS)	**NS (non-smokers)**
Condensed chromatin (CC)	**Nuclear-cytoplasmic (N/C)**
Differentiated cells (DIFF)	**polycyclic aromatic hydrocarbons (PAH)**
Dual smokers waterpipe smokers and cigarette smokers (DS)	**pyknotic cells (PYC),**
Feret ratio (F/R)	**Pyknosis (PYK)**
International Agency for Research on Cancer (IARC)	**Total number of micronuclei (TMN)**
Karyorrhexis (KR)	**United Arab Emirates (UAE)**
Karyolysis (KL)	**Standard deviation (SD)**
Karyolitic cells (KYL)	**Waterpipe tobacco smokers (WTS)**
Kingdom of Saudi Arabia (KSA)	**World Health Organization (WHO)**

*The preferred reporting items for systematic reviews and meta-analyses protocols (PRISMA-P)

ABSTRACT

Introduction: Water-pipe tobacco smoking (WTS) is a form of tobacco use with different names. There is a misconception that passing tobacco smoke through water reduces its harmful effects to increase its popularity. One million individuals smoke water-pipe daily, resulting in approximately five million deaths per annum globally. The toxic effects of WTS are related to the several components of the tobacco mixture. WTS contains 100 times more tar, four-fold more nicotine, eleven-fold more Carbon Monoxide (CO), and two to five-fold more polycyclic aromatic hydrocarbons than cigarettes.

WTS is linked with adverse health consequences such as respiratory malignancies and cardiovascular diseases. Toxins released from WTS result in multiple genetic changes inside human cells leading to disruption in the cell cycle, dysregulation in cell signalling and DNA damage. These events can be monitored in the saliva, oral mucosa, blood, urine, and exfoliated using biomarkers. In 2015, the World Health Organization (WHO) published an advisory note urging the global research community to focus on the health effects of WTS, the need for more understanding of the biological responses of WTS on human cells and establishing standardized biomarkers of exposure and effect.

Methods and analysis: This systematic review aimed to determine the cellular and molecular biomarkers that reflect the toxic outcome of water-pipe tobacco smoking compared to cigarette smoking on the oral mucosa and saliva in adults and adolescents. The review included case-control, cohort and cross-sectional studies published between 2000 and August 2020. Clinical trials, RCTs, Case reports, reviews, letters to the editor, conference abstracts, and cell and /or animal studies were excluded. Four electronic databases were searched (PubMed, Science Direct, EBSCOHost, and Scopus), grey literature was not searched. The search was limited to English and Arabic scientific articles only.

Two reviewers screened and completed the study eligibility and data extraction forms independently. Any disagreements were resolved through discussion or with a third reviewer. When necessary, authors of published articles were contacted for missing or additional data for

clarification. Eligible studies were critically appraised by two independent reviewers using standardized critical appraisal tool; the results were reported in narrative form and tables.

Results: There were 12 included reviewed studies with 1379 participants. The overall evaluation of the risk of bias suggested four studies as a high risk ,five studies as moderate risk, two as a low risk, and a final one scored as very low risk. Cellular and molecular biomarkers that were found to detect the carnocigencity and the nuclear changes of WTS on the oral mucosa and saliva were as follow: micronuclei(MN), pyknosis (PYK), karyorrhexis (KR), karyolysis (KL), broken eggs (BE), repair index, binucleated cells (BN), basal cells (BC), nuclear buds (NB), differentiated cells (DIFF), condensed chromatin (CC), nuclear size, the nuclear-cytoplasmic ratio (N/C), feret ratio (F/R), Cytoplasm Size, DNA tail moment, DNA tail length, p53, finally CYFRA21-1 and 8-OHdG did not correlate with WTS. At the meta-analysis five forest plots display the mean of micronuclei in 1000 cell, the overall mean difference was 5.73(95% CI: 2.41 to 9.05) P=0.00, I^2 = 98.66% between the WTS group and NS, 1.05(95% CI: -0.29 to 2.39) P=0.18, I^2 = 39.15% between the WTS group and CS group, 6.24(95% CI: 1.88 to 10.61) P=0.00, I^2 = 97.17% between the CS group and NS group , 13.89 (95% CI: 10.09 to 17.70) P=0.00, I^2 = 92.82% between the dual smoking group and NS group, 5.05 (95% CI: 3.75 to 6.35) P=0.73, I^2 = 0.00% between the dual group and WTS.

Conclusion: The use of WTS and CS compromises human health, especially the human oral cavity. The present study highlighted the impact of water-pipe smoking and cigarette smoking on active tobacco users' oral mucosal cells and saliva. For instance, although biomarkers are used to analyze nuclear damage, the reported outcomes and comparison of studies did not indicate which consumed product, WTS, or CS as a more significant carcinogenic effect. There is a need for further research that explores oxidative stress, nuclear changes and DNA adduct biomarkers in WTS.

Key words: cellular biomarkers, molecular biomarkers, oral mucosa, water-pipe tobacco smoking, systematic review

1. INTRODUCTION

Tobacco consumption is a universal problem due to its popularity and adverse effects on the human body. Tobacco is available in several forms and can be smoked, snuffed, or chewed (Hessami *et al.,* 2020). WTS is the most predominant form of tobacco use among young adults in Middle Eastern countries. It is the second most predominant type in other countries after cigarettes (Akl *et al*., 2011; Maziak *et al*., 2021).

Epidemiology

According to Wolfram *et al.* (2003), millions of individuals smoke water-pipe tobacco daily, resulting in approximately five million deaths per annum globally (Neergaard *et al.,* 2007). If current tobacco regulations and usage rates persist, scientists expect an increase in the mortality rate beyond one billion individuals per year (Zafeiridou *et al.,* 2018; Chugh *et al.,* 2020; Giovino *et al.,* 2012). In addition, a cohort study involving 50,000 participants showed that WTS is associated with early death and increased mortality in the Middle Eastern communities (Etemadi *et al.,* 2017). These results were supported in a systematic review conducted in 2017, where the highest prevalence of WTS use was reported in Eastern Mediterranean and European countries among younger individuals. Of interest was the exponential increase of WTS use in North America and Europe over the last two decades (Waziry, 2017). Furthermore, a Global Tobacco Survey conducted in thirteen low, and middle-income countries documented a higher use among Russian and Ukrainian women than their male counterparts (Palipudi *et al.,* 2012).

WTS mechanism

Waterpipe tobacco smoking (WTS) is a form of tobacco use with different names (shisha, nargileh, hookah and hubble bubble), in which tobacco is heated on burning charcoal, the resultant smoke

passeD through water and a hose covered by aluminium foil to be inhaled through a mouthpiece. In this type of smoking, the tobacco is ground into a paste and mixed with flavourings. The paste comprises 5-10% of shredded tobacco leaves mixed with honey and molasses (Javed *et al.,* 2019; Vallès *et al.,* 2018; Jacob *et al.,* 2013). Over 250 flavours (e.g. chocolate, mint, and fruits) are available to add to the tobacco mixture to increase its acceptability and popularity among females and the younger population. However, a distinction must be made between the standard WTS and the electronic WTS (e hookah), which also comes in different flavours, but charcoal is absent from its nicotine delivery system (Schubert et al., 2013; Zaatari et al., 2019).

Hakim Abul Fath, the physician who invented the WTS during Emperor Akbar's reign (1556 to 1605), suggested that passing tobacco smoke through water reduced its harmful effect. Ever since, this misconception initiated several international studies comparing the use of WTS with other forms of smoking (Gupta *et al.,* 1992; Chattopadhyay *et al*., 2000). Despite its historical origin in India, WTS is widely practised as a cultural activity in Middle Eastern areas (Javed *et al.,* 2017).

WTS toxicity

The toxic effects of WTS are related to several components of the tobacco mixture, including the tobacco itself, charcoal and flavorants. Toxins and carcinogens comprise of tobacco-specific nitrosamines, nicotine, polycyclic aromatic hydrocarbons (PAH) (e.g., benzo[a] pyrene, anthracene), volatile aldehydes (e.g., formaldehyde, acetaldehyde, acrolein), benzene, nitric oxide, carbon monoxide (CO) and heavy metals (arsenic, chromium, lead) (Patil *et al.,* 2019). Furthermore, the duration of one water-pipe smoking session is usually longer than the duration of one cigarette smoking session, resulting in more volume of smoke and toxins being inhaled (Zielińska-Danch, 2021).

The International Agency for Research on Cancer (IARC) identified seventy tobacco products as carcinogenic, in which sixteen products are proven to affect human cells (Napierala *et al*., 2016; Talhout *et al.*, 2012). Some of the toxic ingredients such as polycyclic aromatic hydrocarbon (PAH) result from the burning charcoal while products such as carbon monoxide (CO) originate from the fabrics of the hose (e.g. leather and plastic) (Monzer *et al.,* 2008; Saleh and Shihadeh 2008).

The carcinogenic effect of PAH is seen in human epithelial cells in the oral cavity and larynx, which result in higher amounts of DNA adduct in smokers compared to non-smokers (Jacob *et al*., 2011). Furthermore, an experimental study identified an association between the level of nitrosamine products in the body and an increased risk of developing nasal mucosal tumours. Nonetheless, these products were reported in the urine of smokers and non-smokers who were exposed to tobacco smoke (Zhang *et al.,* 2009; Lee *et al.,* 2008).

Apart from toxins, WTS contains 100 times more tar, 4-fold more nicotine, 11-fold more CO, and 2 to 5-fold more polycyclic aromatic hydrocarbons than cigarettes (Jacob *et al.,* 2013). Additionally, the habitual use of WTS with simultaneous alcohol consumption results in the release of more toxins than the use of WTS alone (Leavens *et al.,* 2020).

WTS health implications

WTS is associated with adverse health outcomes such as malignancies, respiratory and cardiovascular diseases and can lead to complications during pregnancy (Awan *et al.,* 2017). In 1999, three instances of keratoacanthoma were reported in water pipe smokers; these findings raised suspicion of the possibility of WTS as a predisposing factor of cancer (El-Hakim *et al.,* 1999). In addition, a study conducted by Dar *et al.* (2012) in Kashmir, India, documented a total of 702 oesophagal squamous cell carcinomas in water-pipe smokers. Moreover, an association was recorded between the socio-cultural behaviour of water-pipe tobacco smokers in Arab countries and the incidence of cancer of the lower lip, tongue and floor of the mouth (Al-Jaber *et al.,* 2016).

In a review, Prior *et al.* (2017) documented eight studies investigating the effects of WTS on the oral cavity. These studies focused on the role of WTS and its components on oral health. The results suggested that WTS could be implicated in several health problems, including head and neck malignancies (Prior *et al.,* 2017). However, there is a paucity of studies investigating the direct relationship between WTS and malignant oral tumours. This is in contrast to the number of investigations published reporting the relationship between cigarette smoking and oral cancer (Llewellyn *et al.,* 2004; Nayak *et al.,* 2012; Chher *et al.,* 2016; Ramoa *et al.,* 2017 and Javed *et al* 2017).

Other health implications that arise from short term water-pipe smoking, i.e., one hour per day for seven days, include but is not limited to airway obstructions and lung disease, neutropenia, raised

pro-inflammatory cytokines and oxidative stress markers (Khabour *et al.,* 2012). An additional negative health outcome of WTS is hypoxia, resulting from CO poisoning due to the irreversible conjugation of CO with blood haemoglobin. The disorder's most common signs and symptoms vary from loss of consciousness, dizziness, headache, and vomiting (Eichhorn *et al.,* 2018; Münzel *et al.,* 2020).

Cellular and molecular biomarkers

A biomarker is a characteristic indicator of biological and pathogenic processes and is measured by several molecular techniques. This biosignature reflects the pharmacological outcomes of treatment intervention, detects exposure and cellular responses to carcinogens, and cellular liability to toxic substances. Various biomarkers were reported in studies, including genomic, oncogenic, oxidative stress and immunologic biomarkers (Rothman *et al.,* 1995; Tanaka *et al.,* 2011).

In biology, principally in biochemistry, a molecule defined as any tiny particle for example charged organic molecules or to substances (called biomolecules) produced and occur naturally in living organisms such as proteins, carbohydrates, DNA, *et***c.** While ,a cell defined as the basic unit for life ,consists of a ctoplasim enclosed within a membrane including many biomolecules such as proteins and nucleic acids.

Toxins released from WTS result in multiple genetic changes inside human cells, leads to disruption of the cell cycle, dysregulation in cell signalling and DNA damage. These events can be monitored in saliva, oral mucosa, blood, urine, and exfoliates using biomarkers (Ezera *et al.*, 2020).

According to the Institute of Medicine, Committee biomarkers are classified into four groups: external exposure measurements, internal exposure biomarker, biologically effective dose estimation biomarkers, and potential harm biomarkers (Bondurant *et al.*, 2001; Perera 1987). Potential harm biomarkers include non-functional effects on cells. The cells investigated using these biomarkers of potential harm could act as surrogate markers and be used to determine actual harm, pre-clinical and clinical studies or diseases. In this study, we will focus on the biomarkers of potential harm.

Biomarkers can be used to detect the nuclear changes in the buccal mucosa through non-invasive techniques that help establish the cytological studies that evaluate the carcinogenic effect on the

cell before clinical signs of cancer appear (Stich 1984). Micronuclei (MN) are the most frequently used biomarkers to record the genetic alteration in the cells exposed to carcinogens (Palaskar *et l*, 2010). Nevertheless, using additional biomarkers such as Karyorrhexis (KR), Karyolysis (KL), Pyknosis (PYK), broken eggs (BE), and the repair index may lead to a better understanding of these nuclear changes (Farhadi 2017).

The hazardous effect of WTS can be explained by the formation of inflammation provoked by reactive oxygen species (ROS). These molecules produce oxidative stress that negatively affects DNA, protein, and body lipids (Taati *et al.*, 2020). Additionally, PAH can bind with normal DNA and form DNA adducts, which are considered the first sign of abnormal replication and formation of cancerous cells (Eissenberg and Shihadeh, 2009).

Acute or chronic exposure of mice lungs to WTS can lead to changes in inflammatory cytokines, create oxidative stress markers and increase the numbers of circulating macrophages, neutrophils and lymphocytes (Khabur *et al.*, 2012). All these alterations, besides the DNA adduct, ROS and oxidative damage, are used as a measurement of the carcinogen biological effects, which can be translated as a biomarker (Szyfter *et al.*, 2019; Ewa and Danuta, 2017)

WTS vs. CS hypothesis

Eker *et al.* (2016) reported a significantly higher amount of chromosome aberrations and micronuclei in the blood of exclusive water-pipe smokers than in the blood of non-smokers. Furthermore, Nemmar *et al*. (2017) conducted a study on mice who were forced to inhale water-pipe smoke for six months and showed a two-fold increase in DNA damage in their lung tissues compared to a control group.

Khabour *et al.* (2011) investigated the frequency of sister chromatid exchanges in lymphocytes of WTS, CS and NS and reported the highest frequencies in were found in WTS and CS compared to NS. Furthemore, Alsatari *et al.* (2012) found that the level of chromosome aberrations was 2.7 fold higher in WTS and 3.7 fold higher in cigarette smokers than in non-smokers.

Current studies on WTS are based on inadequate data regarding the frequency and duration of WTS, male domination, and the low figures of exclusive water-pipe smoking (Mamtani *et al.*, 2017; Eissenberg and Shihadeh, 2009).

A water-pipe session lasts for more than 20 minutes and involves an intensive smoke inhalation higher than cigarettes, making it more harmful than cigarette smoking. However, most clinical and experimental studies on WTS provide evidence of increased cellular inflammation and oxidative stress (Shihadeh and Saleh, 2005).

Why it is important to do this review

Given the high rate of WS use among young adults in Middle Eastern countries and its rapid increase in popularity in Western countries, there is an urgent need to identify cellular and molecular biomarkers of oral cancer in this group of individuals.

In 2015, the WHO published an advisory note urging the global research community to focus on the health effects of WTS. The necessity to interpret the biological response of WTS on human cells remains valid. Moreover, there is an increasing urgency to establish standardized biomarkers of exposure and effect (e.g. DNA adducts) to determine whether WTS prompts inflammatory and oxidative stress response or not (WHO Study Group on Tobacco Product Regulation, 2015; Cobb C *et al.*, 2010; Jawad *et al.*, 2013).

It is imperative to compare the hazardous consequences of water-pipe smoking to cigarette smoking. Physicians need to warn their patients about the harmful risks of WTS and inform them that they share the same poor health outcomes and toxicant effects of CS. Moreover, they need to advise and campaign against the misconception that WTS is less harmful than cigarette smoking.

For these reasons, the authors were motivated to conduct a systematic review to analyze studies published in the last 20 years to determine the cellular and molecular biomarkers in water-pipe tobacco smoking that may have carcinogenic effects on the oral cavity.

A preliminary search of PROSPERO, MEDLINE, the Cochrane Database of Systematic Reviews, and the JBI Database of Systematic Reviews and Implementation Reports was conducted, and no current or ongoing systematic reviews on the topic were identified.

1.1 Research question

What are the cellular and molecular biomarkers detected in the oral mucosa and saliva in water-pipe tobacco smokers compared to cigarette smokers in adults and adolescents?

1.2 Objectives

This study aimed to revise and analyze literature and selected studies that determined the cellular and molecular biomarkers that reflect the toxic effect of water-pipe tobacco smoking on the oral mucosa and saliva and compared it to cigarette smoking in adults and adolescents.

1.3 Table 1: PECO

Population(P)	Adults and adolescents
Exposure (E)	Waterpipe tobacco smoking
Comparison (C)	Cigarettes smoking
Outcomes (O)	Primary outcome: Cellular and molecular biomarkers in the oral mucosa and saliva in WTS. Secondary outcome: Cellular and molecular biomarkers in the oral mucosa and saliva in WTS. compared to non-smokers and/or cigarette smoking.

2. METHODS

2.1 Protocol registration

The review was conducted following the requirements contained within the PRISMA-P checklist for systematic review and meta-analysis. This protocol was registered with the PROSPERO registry of the University of York. The registration number is CRD42020209697 and is based on (PRISMA-P) statement guidelines (appendix 1). Our research was also registered with the BMREC at the Western Cape University Registration number: BM20/9/4.

2.2 Eligibility criteria

The eligibility was checked and reviewed independently by two reviewers. Conflicts were addressed by discussion or by a third reviewer.

2.2.1 Study types

We included all studies that analyzed the effect of WTS on oral mucosa or saliva as cellular or molecular biomarkers, comparing them to non-smokers and/or cigarette smoking and/or another type of tobacco smoking and/or non-smokers in adults or adolescents. Studies published in English and Arabic between 2000 and August 2020 were included in the review This date was chosen after a preliminary search revealed that no scientific papers on WTS were published before 2000. Cohort, case-control and cross-sectional studies were considered for inclusion. Case reports, reviews, letters to the editor, conference abstracts and cell and animal studies were excluded.

2.2.3 Types of participants

We included studies that evaluate the effects of hookah smoking in adults and adolescents irrespective of their gender.

2.2.4 Types of exposures

The effects of WTS in saliva and on oral mucosal cells was evaluated. The control or comparative groups included are water-pipe tobacco smokers, cigarettes smokers and non-smokers.

2.2.5 Outcome

The primary outcome was to present cellular and molecular biomarkers detected on the oral mucosa and saliva in WTS and to evaluate the salivary and oral mucosal changes detected by

biomarkers of malignant change. The secondary outcome was to compare the changes observed in the primary outcome to those in the control and comparative group. In this research, we excluded the histological change for WTS The research PECO is described in (Table 1)

2.3 Information sources

Four electronic databases were searched (PubMed, Science Direct, EBSCOHost, and Scopus) for published articles. When necessary, authors were contacted if additional information was required.

2.4 Search strategy

An initial search was conducted using the text words in the titles, abstracts, and keywords to develop a complete search strategy. An example of a search strategy outlined for PubMed was undertaken using the keywords: biomarkers, oral mucosa, buccal mucosa, saliva, water-pipe tobacco smoking (shisha, hookah, narghile, arghile, hubble bubble) see table (2).

The search strategy was adapted for each included database. Studies that referred to oral mucosal cells and saliva, biomarker, water-pipe tobacco smokers, cigarette smokers and non-smoker and restricted to English and Arabic language was included. The reference lists of all studies included in the final review were hand-searched for further studies. The search strategy was conducted in duplicate and independently between two authors and the results documented in an Excel spreadsheet.

Table 2: PubMed search strategy

Keywords: biomarkers, oral mucosa, buccal mucosa, saliva, water-pipe tobacco smoking (shisha, hookah, narghile, arghile, hubble bubble	Combinations of MeSH terms and free-text words with Boolean operators as following Search: (((((((((biomarkers) AND (oral mucosa)) OR (buccal mucosa)) OR (saliva)) AND (waterpipe tobacco smoking)) OR (shisha)) OR (hookah)) OR (narghile)) OR (arghile)) OR (hubble bubble) ((((((((((("biomarker s"[All Fields] OR "biomarkers"[MeSH Terms]) OR "biomarkers"[All Fields]) OR "biomarker"[All Fields]) AND ((((("mouth mucosa"[MeSH Terms] OR ("mouth"[All Fields] AND "mucosa"[All Fields])) OR "mouth mucosa"[All Fields]) OR ("oral"[All Fields] AND "mucosa"[All Fields])) OR "oral mucosa"[All Fields])) OR ((((("mouth mucosa"[MeSH Terms] OR ("mouth"[All Fields] AND "mucosa"[All Fields])) OR "mouth mucosa"[All Fields]) OR ("buccal"[All Fields] AND "mucosa"[All Fields])) OR "buccal mucosa"[All Fields])) OR ((("saliva"[MeSH Terms] OR "saliva"[All Fields]) OR "salivas "[All Fields]) OR "saliva s"[All Fields])) AND ((((("tobacco, waterpipe"[MeSH Terms] OR ("tobacco"[All Fields] AND "waterpipe"[All Fields])) OR "waterpipe tobacco"[All Fields]) OR ("waterpipe"[All Fields] AND "tobacco"[All Fields])) AND (((((((("smoke"[MeSH Terms] OR "smoke"[All Fields]) OR "smoke s"[All Fields]) OR "smoked"[All Fields]) OR "smokes"[All Fields]) OR "smoking"[MeSH Terms]) OR

	"smoking"[All Fields]) OR "smokings"[All Fields]) OR "smoking s"[All Fields]))) OR ((("smoking water pipes"[MeSH Terms] OR (("smoking"[All Fields] AND "water"[All Fields]) AND "pipes"[All Fields])) OR "smoking water pipes"[All Fields]) OR "shisha"[All Fields])) OR ((((("smoking water pipes"[MeSH Terms] OR (("smoking"[All Fields] AND "water"[All Fields]) AND "pipes"[All Fields])) OR "smoking water pipes"[All Fields]) OR "hookah"[All Fields]) OR "hookahs"[All Fields])) OR ((((("smoking water pipes"[MeSH Terms] OR (("smoking"[All Fields] AND "water"[All Fields]) AND "pipes"[All Fields])) OR "smoking water pipes"[All Fields]) OR "narghile"[All Fields]) OR "narghiles"[All Fields])) OR "arghile"[All Fields]) OR (("hubble"[All Fields] OR "hubble s"[All Fields]) AND ((((("bubble"[All Fields] OR "bubble s"[All Fields]) OR "bubbled"[All Fields]) OR "bubbles"[All Fields]) OR "bubbling"[All Fields]))

2.5 Data collection and analysis

2.5.1 Study selection

Potentially relevant studies were retrieved and their citation details imported into RAYYAN QCRI (Ouzzani et al., 2016), where duplicates were removed. Two reviewers (authors DE and TR) independently selected, screened, and completed the study eligibility and data extraction forms following a pilot study. The process proceeded in two phases. During the first phase, the article's title and abstract were read independently by two reviewers. The article titles and the abstracts that qualified for inclusion and potentially suited were selected for further review. Following this, the full texts of qualifying articles were read. The two primary reviewers discussed any disagreements during the selection process. A third reviewer (FKD) served as an arbitrator if no agreement is reached. Studies that did not meet the inclusion criteria were excluded, and the reasons for exclusion were reported. The search results and the study inclusion process was reported in full in the final systematic review and presented in a Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) flow diagram.

2.5.2 Data extraction and management

The two primary reviewers (authors DE and TR) independently extracted and recorded information on a data extraction sheet customized on an Excel sheet. Any disagreements between the reviewers were resolved by discussion or with a third reviewer (FKD). When necessary, the authors of the original manuscript were contacted to clarify or request missing or additional information.

2.5.3 Data items

The extracted data were recorded on an Excel spreadsheet and included:

- Study details: author, year of study country, study design and inclusion criteria for the study.
- Population Details: number of participants, age, gender, duration and consumption of WTS. use
- Sample population groups (groups of comparison): non-smokers, water pipe tobacco smokers, cigarettes smokers, and other types of smoking

- Biomarkers such as micronuclei, karyorrhexis karyolysis, condense chromatin, P53and DNA aberration.
- Sample type: saliva, exfoliated cells, biopsy
- Analysis method (laboratory technique).
- Outcomes

2.6. Quality appraisal

The JBI Critical Appraisal Checklist for cross-sectional, case-control and cohort studies was used to evaluate the methodological quality and risk of bias of studies included by answering eight questions with Yes, No, Unclear and Not applicable. In the following parameters, the inclusion criteria of the included studies, the study subjects and settings, the validity and reliability of the exposure measurement, strategies used to identify the confounding factors, the validity and reality of the outcome measurements and the suitability of the statistical analysis. Two reviewers (authors DE and TR) assessed the studies independently, and a third (FKD) acted as a mediator to resolve disagreements that could not be resolved by discussion. The critical appraisal results were reported in narrative form and a table. Regardless of their methodological quality, all studies were subjected to data extraction and synthesis (where possible).

2.7 Data synthesis

After critical appraisal of all studies, construction of data summary tables was performed by grouping similar outcomes and similar studies together (similarity in study design, gender, age, comparing groups). Finally, the outcomes were synthesized as a comprehensive narrative discussion, and the results were presented in tables and figures where possible.

The homogenous outcomes were synthesized as quantitative evidence in the form of meta--analysis reported in STATA version 17 (StataCorp LLC., College Station,TX) (StataCorp 2017; Mahmassani,et al 2021). Assuming that our data were heterogeneous, a random-effects model for meta-analysis was used. A forest plot was generated graphically in a meta-analysis to represent relative risk and 95% confidence intervals.

The effect measurement was the mean difference with 95% (CIs) for continuous data. Different scales were modified into a standard metric unit (standardized mean differences with 95% CIs) to facilitate the comparability between the studies and to define the effect size.

To assess the heterogeneity of the included studies, a chi-squared and I^2 test was used; a result lower than 50% of I^2 was considered no heterogeneity, a result higher than 50%of I^2 was considered as high heterogeneity. A sensitivity analysis and subgroup analysis were not done due to the high heterogeneity of the included studies and the few numbers of studies involved in the meta-analysis.

2.8. Reporting bias assessment

If there were more than ten included studies at the meta-analysis (Lam et al., 2020), publication bias should be demonstrated in a funnel plot, but our meta-analysis included only four studies.

2.9. Confidence in cumulative evidence

If we had enough studies included in the meta-analysis (homogenous results), we were planning to use Grading of Recommendations Assessment, Development and Evaluation (GRADE) for assessment of evidence (Guyatt *et al.*, 2008), but our meta-analysis included only four studies.

3. Results

3.1 Study selection

The initial search of the four databases as presented in section 2.3 resulted in 4542 studies,then uploaded into RAYYAN QCRI (Ouzzani et al., 2016), of which 3134 were duplicates that had been removed. Independently, the two primary reviewers (DE and TR) screened 1391 studies by their titles and abstract; the screening revealed seventeen matched studies with the inclusion criteria. After reviewing full-text studies, six studies were excluded due to irrelevant content. The two primary reviewers discussed any disagreements during the selection process. A third reviewer (FKD) served as an arbitrator if no agreement was reached. An additional search of the references of the eligible studies provided one additional study. In conclusion, twelve studies were included in this research. The study selection process is illustrated as a flow diagram based on preferred reporting items for systematic reviews and meta-analyses PRISMA-P 2020 (Figure 1).

Included studies

The final review included twelve studies Amer (2018); Azab(2015); Dehghan (2020); Eker (2016); El-Setouhy (2008); Jar-Allah (2014); Naderi (2017); Prasad (2019); Rajabi-Moghaddam (2020); Seifi (2013); Silveira (2017); Taghibakhsh (2019), involved a total of 1379 participants (541 nonsmokers, 511 water-pipe tobacco smokers, 211 cigarettes smokers, 116 cigarettes and water-pipe tobacco smokers). There was substantial heterogeneity between studies in respect of the group of comparison, inclusion criteria, exclusion criteria, sample types, laboratory analysis methods and outcomes.

Excluded studies

There were six (n= 6) excluded studies (Table 3)

Table 3: Table of Excluded studies

Study citation	Reason for exclusion
Ali (2007)	The study aimed to record the oral effect of takhzeen al-qat, WTS and cigarette smoking at the histological level.
Bacha (2007)	The design of the study was unclear. Cotinine measured the degree of addiction which was considered as a biomarker of exposure but not the carcinogenic effect of WTS
Alshammari (2017)	The outcome was detected in the blood, not in the oral mucosa or saliva.
Volkove (2017)	The study was conducted among university students with different smoking habits but smoking habits were not categorized.
Zaid (2018)	examined tissue samples from diseased tissue of patient with diagnosed oral cancer.
Arazi (2019)	The outcome was various concentrations of peroxidase (POX) enzymes that catalyze oxidation-reduction reactions and 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) before and after exercise to determine whether exercise affects the concentrations of these enzymes.

Geographic locations of included studies

Eleven of the studies were conducted in the Middle East countries (Iran, Jordan, KSA., UAE and Egypt), only one study was in South America (Brazil). Five studies conducted in Iran (Seifi (2013); Naderi (2017); Taghibakhsh (2019); Rajabi-Moghaddam (2020); DehghanNezhad (2020)), two studies in Egypt; El-Setouhy (2008); Amer (2018), one in Turkey; Eker (2016),one study in Jordan; Azab (2015) , another one study in Brazil; Silveira (2017), one in K.S.A.; Jar-Allah (2014), and the last study in UAE Prasad (2019).

3.2 Study Characteristics

All the extracted data of the studies characteristcs are included in Table (4) and (5) .

The study characteristics for all the selected 12 articles are as follows.

The investigation of genetic toxic effects and damage to buccal mucosal cells of water-pipe smokers were conducted by using the micronucleus assay

sample collection technique. Genetic toxicity levels depended on the frequency of water-pipe smokers hence these participants showed statistically significant micronuclei count compared to non-smokers (Dehgham Nezhad, 2020). The effect of smoking on the oral cavity, as determined by Prasad (2019), was evaluated using two different staining techniques: the Feulgen and Acridine orange to count the frequency of micronucleated cells. In the study of causative DNA damage among active water-pipe smokers and specifically in the peripheral blood leukocytes and buccal cavity, Al-Amrah (2014) discussed the DNA damage using the comet assay technique to collect smoke condensate. As a result, several chemical compounds in the smoke condensates especially (Jurak and Moassel) ,which are causing more damaging effect to the buccal cavity and blood leukocytes of water-pipe smokers than the other chemicals. However, the micronuclei (MN) evaluation of active water-pipe smokers, according to the study of El-Setouhy (2008), by a modified Papanicolaou method, showed statistical significance in the MN of oral cells obtained from waterpipe smokers and non-tobacco smokers. The possible malignant alteration experienced by water-pipe smokers, especially in the oral mucosal epithelia, was determined by studying the nuclear-cytoplasmic ratio (N/C), Feret ratio (F/R), percent of karyorrhexis (KR), vacuolization of cytoplasm, two or multilobed nuclei, inflammation and candida was investigated by Sefie (2013),

using the Papanicolaou technique. For water-pipe smokers, the N/C and F/R to were lower compared to the increase in cytoplasm size of water-pipe smokers. In the case of percent of KR, vacuolization of cytoplasm, two or multilobed nuclei, there was a lack of statistical significance reported in the lateral surface of the tongue, buccal mucosa and floor of the mouth of smokers. A statistical significance was observed for both inflammation and candida of water-pipe smokers compared to normal individuals. Amer (2016), compared the oral mucosa of three smokers: water-pipe smokers, cigarette smokers, mixed smokers, and non-smokers. Saliva samples of these smokers and non-smokers were analyzed using ELISA kit and mucosal biopsy by the H&E technique. The outcome showed p53 was statistically significant between the smokers' groups and non-smokers group. However, the CYFRA 21-1 levels were not significant for all smokers and non-smokers. According to Eker (2016), chromosome breakage in water-pipe smokers due to genotoxic effects and mutations was observed for active and frequent water-pipe smokers, especially in the fragment ratio, gap, micronucleus and binucleus parameters hence suggesting that water-pipe and, in this case, hookah causes genotoxic effects. A comparison study by Naderi (2017) determined the relationship in the cytotoxic effects on human buccal mucosa cells of water-pipe smokers and cigarette smokers. The study focused on the analysis of pyknosis, karyorrhexis and karyolysis of these two categories of smokers. The statistical results varied such that the KL and PYK were statistically significantly different for both water-pipe and cigarette smokers.

In contrast, there was a lack of statistical significance in the karyorrhexis analysis of water-pipe and cigarette smokers. Also, cytotoxic effects were a function of exposure and dosage for water-pipe smokers, unlike cigarette smokers with comparatively higher cellular death than water-pipe smokers. In studying the cytogenetic biomarkers using Papanicolaou technique, Rajabi-Moghaddam (2020), reported that even though the mean number of MN in water-pipe smokers and cigarette smokers did not differ significantly, the water-pipe smokers showed higher levels of MN compared to cigarette smokers. For this reason, the genotoxic effect was significantly higher in water-pipe smokers than in cigarette smokers.

Taghibakhsh (2019), focused on smokers of water-pipe, cigarettes and tobacco, especially their susceptibility to oral cancers and dysplastic lesions. The analysis of cell percentage was conducted in the MN, KR, KL and BE in the buccal mucosa. The result showed statistical significance in the percentage of cells found with micronucleus, karyorrhexis, karyolysis, and broken egg in the

buccal mucosa of hookah users compared to non-hookah users, i.e. in the control group. The Silveria (2017), study investigated cancer risks among water-pipe smokers by analyzing the cellular changes such as pyknotic cells (PYC), karyolitic cells (KYL), karyorrhetic cells (KHC), condensed chromatin (CC), binucleated cells (BN), basal cells (BC), nuclear buds (NBUD) and differentiated cells (DIFF). The study reported that the CC did not reveal any statistical significance compared with other cells. This implies that the cell cycle interruption among water-pipe smokers was attributed to DNA damage with a higher risk of cancer exposure. Azab (2015) study focused on genotoxicity in the saliva, urine, and serum of water-pipe smokers and healthy non-smokers. In using a biomarker such as 8-OHdG the reported variation between water-pipe smokers and non-smokers suggested that 8-OHdG is not an appropriate biomarker to determine the genotoxicity of water-pipe smoking among active water-pipe users. This is because of the lack of correlation and levels of 8-OHdG for all samples.

Overview of the participants

This section presents the characteristics of participants in the selected reviewed articles. Specifically, the participants' gender, age, and socio-economic status are presented and discussed where available.

The largest number of participants was 400 (Prasad 2019), who were divided into four groups. In the other hand, two studies only mentioned the economic status of the participants (Azab 2015 and Al-Amrah 2014).

The demographic distribution of participants in the study conducted by Azab 2015 focused on healthy adults with smoking habits, specifically, water-pipe smoking. In total, 76 smokers adults were included in the study and forty-six were non-smokers. The gender distribution showed that thirty-four males and thirty-two females constituted the 76 smokers while the 46 non-smokers accounted for equal numbers of males and females. The average age of smokers and non-smokers was 30 and 31 years, respectively. (Al-Amrah 2014) divided the smokers particepants according to their socio-economic status in to two groups ,differed on an income basis such that the monthly

salary, fifty-eight of smokers had a monthly salary less than 990 Jordanian Dinar (JD), fourty - nine of them had a monthly salary more than 990 JD.

The selected participants had three distinct categories with thirty individuals in each category and were only males. The categories of participants were water-pipe smokers, cigarette smokers and non-smokers. The average age of all participants is 21 years old. The smokers were categorised by smoking frequency, i.e. light, moderate or heavy smoking. In selecting the participants, the health status was determined to ensure non-health-related diseases such as diabetes or oral lesions exist among the participants (Rajabi-Moghaddam 2020)

The study by Taghibakhsh (2019) consisted of seventy-two male hookah and non-smokers equally distributed into two groups: a control group of thirty-six and hookah smokers of thirty-six participants. The sampling was conducted in a Hookah café in Tehran. Participants' selection criteria for this study were no history of smoking (for the control group only), alcohol and drug consumption, head and neck radiotherapy, or chemical exposure. In terms of gender, all participants were male and assumed to belong to similar socio-economic status considering the location of the café that the selected participants visited.

In the cross-sectional study by Silveira (2017), the participants were paired according to age, gender, and frequency of alcohol consumption. The water-pipe smoker group included forty participants, and the control group included forty non-smokers. The selection criteria ensured that participants did not have pre-existing health complications such as cancer or any chronic diseases. In addition, the group consisting of water-pipe smokers had smoked at least 60 minutes within 24 hours and for an average of 1–2 days per week for at least one year.

Eker (2016), studied the effects of hookah smoking on chromosome aberration and micronuclei ratio of hookah smoking individuals. The participants included in this study were thirty individuals who had smoked hookah an average of two times a week and for at least one year. A control group of thirty participants included in this study did not smoke hookah or cigarettes. The overall average

age of participants, i.e. hookah smokers and non-hookah smokers, was 21.5 years old, and all participants were of similar gender.

In the study of Amer (2016), sixty-four participants consisted of sixteen per group, namely: (a) nonsmokers; (b) shisha smokers; (c) cigarette smokers; (d) dual smokers cigarette and shisha smokers. The participants were certified to be healthy, and no participants had any apparent oral lesions. Potential participants who had any systemic conditions or were exposed to any other carcinogenic substance were excluded from the study. In each category, the selection criteria differed according to the smoking frequency. For example, for shisha smokers, the frequency of shisha smoking was at least three times in seven days and over a period not less than five years. In cigarette smokers, cigarette smoking frequency must have been at least ten cigarettes daily and for at least five years.

The El-Setouhy (2008), study utilized a random sampling approach that included 2358 households from 9 villages located in the Qalyubia governorate, Egypt. A total of 206 participants were identified in the study. The eligibility criteria included both age and gender; hence the minimum age of the participants was 18 years and predominantly male because only 1.2% of rural women in that area smoked at the time of the investigation. The criteria for selecting water-pipe smokers were as follows: they needed to have smoked every week while cigarette smokers needed to have consumed a minimum of 100 cigarettes. The third category of participants in this study were non-smokers. A total of 128 participants considered current water-pipe smokers, and a control group of 78 participants who had never smoked between 2004–2005 were identified and selected. Participants' demographic data included age, marital status, educational status, and occupation. Also, the smoking habit and the frequency of water -pipe smokers on a daily and weekly basis was considerd. In addition, other criteria for selecting smoking participants included symptoms of nicotine dependence, including quitting behaviour, degree of inhalation of tobacco smoke and the current exposure to occupational chemicals and pesticides, and participants' body weight and height.

In the study by Prasad (2019), a combined population of Arab and Indian nationalities were investigated. The selected nationalities in this study engaged in Waterpipe Tobacco smoking of shisha, cigarette and non-smokers. The toxic effect on oral mucosa at a cytogenetic level was

examined for the smoking participants. There were four categories of participants; shisha and cigarette smokers combined, shisha smokers, cigarette smokers and non-smokers. Each category consisted of 100 participants each. Although the study had male and female participants, the majority was male. Eighty-nine percent of the Shisha smokers were male, so were all the cigarette smokers. Ninety per cent of the participants who smoked both Shisha and cigarette smokers were male, and so was 88% of the control group.

Twenty healthy water-pipe (jurak or moassel) smoking males aged between 28 and 65 with a similar socioeconomic status were included in Al-Amrah's (2014) investigation. The participating water-pipe smokers required a smoking frequency of up to 4 times a day without smoking cigarettes. Further inclusion criteria included no alcohol consumption nor taking any medication. The twenty water-pipe smokers were matched with 20 non-smokering participants.

Naderi (2017), compared the cytotoxic effect of cigarette and water-pipe smoking on the human buccal mucosa among fifty cigarette and water-pipe smokers (twenty-five participants each and a control group of twenty-five). The seventy-five participants were all Iranian males aged between twenty-five and fifty years old. The participants were all water-pipe smokers recruited from a local Water Pipe Café. Potential study participants who were exposed to radiography within six months of the study, who consumed drugs and suffered from systemic disease, were excluded from the study. Lastly, the water-pipe smokers' group included participants whom neither smoked cigarettes nor only smoked 100 cigarettes in their entire life.

DehghanNezhad (2020) conducted his case-control study using a simple sampling technique. The study consisted of thirty non-smokers and thirty water-pipe smokers (total sixty) males between twenty and fifty years old. Potential participants below twenty years of age, those with systemic disorders, oral lesions, or exposed to dental radiography in the previous six months, and alcohol consumers were excluded from the study's smoking and control groups.

Sefie (2013), used a non-probability sampling technique to select 120 Iranian males, in a ratio of forty smokers, forty water-pipe (specifically hookah) users, and a control group of forty non-

smokers. The selected smokers and water-pipe users comprised thirty-eight individuals who used different cafes or entertainment centres and two dental students. The ages of participants were matched with minimum and maximum ages varying between twenty years and forty years old, respectively. Eligible participants, smokers, water-pipe users and non-smokers, were required not to have consumed alcohol and were free of systemic disease. In addition, to be eligible as a non-smoker, individuals were required to be free of smoking history and water-pipe use.

Overview of Exposure types

In El-Setouhy (2008) study, the data collection tool used for participants was a questionnaire to measure the frequency of exposure. The administered questionnaire measured the following: frequency of water-pipe smoking, number of Hagar per week and day, age of smoking initiation, duration of smoking by years and daily minutes, and inhalation of tobacco smoke. The results were tabulated with the first table denoting the total micronuclei (TMN) between WTS and NS by demographics and occupational variables. Also, a tabular analysis of smoking behavior among participants especially water-pipe smoking was determined using the Mann-Whitney Test.

In the study reported by Seifi (2013), the frequency of exposure was measured and analyzed using the inclusion criteria for smokers. The inclusion criteria for cigarette smokers included ten or forty cigarettes consumed daily up to fifteen years. As for water-pipe users, the inclusion criteria was water-pipe use twice daily for a minimum and maximum duration of twenty minutes and eighty minutes respectively within 3-5 years.

The study analysis by Al-Amrah (2014) included the analysis of jurak and moassel smoke condensate by GC-MS. The results indicated that moassel smoke had a more damaging effect than jurak smoke reflected by DNA changes in the oral mucosa. The selected participants smoked four times more a day but did not smoke cigarettes.

Prasad (2019) omitted to highlight distinct criteria for frequency of tobacco consumption, particularly the duration of the smoking habits in any of the compared groups. The study was restricted to investigating the toxic effect of WTS, cigarette effect, and the effects of dual smoking (WTS and CS) in one individual. Additionally, the study reported on the socio-demographic characteristics relating to WTS and CS in the United Arab Emirates (UAE).

DehghanNezhad (2020) evaluated the smoking frequency using the following criteria: weekly consumption and duration of water-pipe smokers was recorded as the number of smoking sessions per year. The participants in (Eker 2016) smoked hookah an average of twice weekly and for at least one year.

Taghibakhsh (2019) did not succinctly indicate the criteria for water pipe smokers. The study by Azab (2015) aimed to identify the relationship between dependence score and levels of 8-OHdG in saliva, urine, and serum. Lebanon Waterpipe Dependence Scale was used to determine the level of dependence in WTS. The participants in this study were categorized into light, moderate and heavy smokers. These were according to parameters used to evaluate the smoking behaviour in water-pipe smokers by recording the initial age of smoking, the time of smoking day,week and year, smoking by hours, and the number of heads used per session.

In Naderi (2017) study, the group of water-pipe smokers comprised of participants who neither smoked cigarettes nor only smoked 100 cigarettes in their entire life. A criterion that specified the cigarette smokers group was not clearly defined, but the author mentioned an average duration of cigarette smoking for 20 years.

The group consisting of water-pipe smokers in Silveira (2017) study, needed to smoke at least sixty minutes within twenty-four hours and for an average of 1–2 days per week for at least one year.

Rajabi-Moghaddam (2020), defined his water-pipe smoker's group by those who smoked one session per week for at least one year. To qualify, cigarette smokers needed to have smoked five cigarettes per day at least five years.

On the other hand, Amer (2018), defined his water-pipe smokers' group as smokers who smoked three sessions per week for at least one year, and the cigarette smokers needed to have smoked ten cigarettes per day.

Figure 1: A flow diagram of the selection process based on preferred reporting items for systematic reviews and meta-analyses (PRISMA-P) 2020.

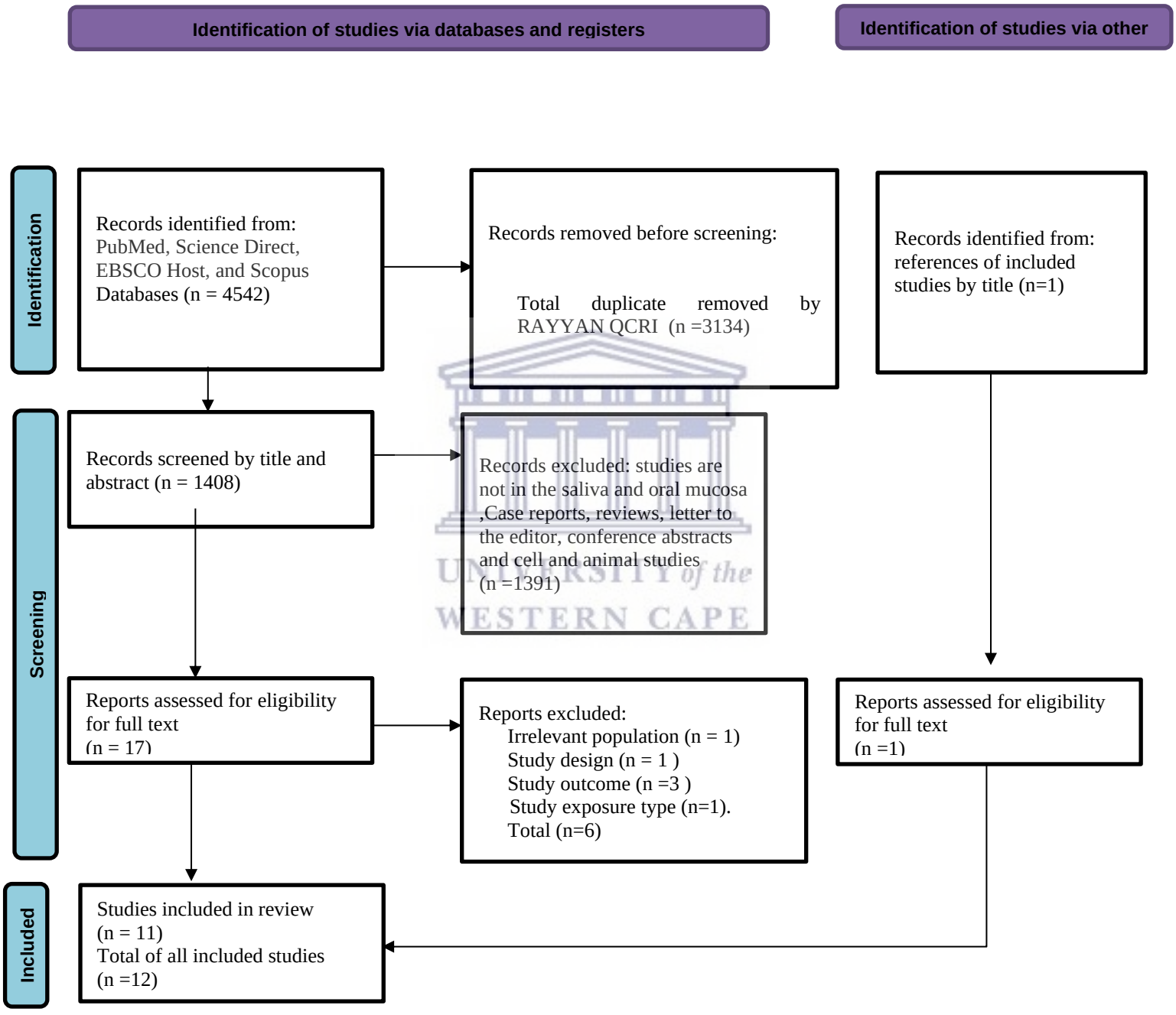


Table 4: Extracted data for the studies and population details.

No	Citation	Country	Study design	Total Number of (P)	Number of (P) in each group	No of Males	No of Females	Age of (P)	Inclusion and exclusion criteria
1	(El-Setouhy 2008)	Egypt	Cross-sectional	206	WTS=128 NS=78	206	0	18 years old or above	Males 18 years old or above/smoked WTS. at least once /week and less than 100 cigarettes in their life NS who never smoked
2	(Seifi 2013)	Iran	Cross-sectional	120	WTS=40 CS=40 NS=40	120 WTS=40 CS=40 NS=40	0	Between 20 to 40 years old	No alcohol s /no systemic disease, no oral lesions/no fixed or removable denture, NS never smoked or exposed to work or home cigarettes smoke. C.S smoke 10-40 cigarettes/day for 6 to 15 years, WTS smoke once or twice/week for 20-80 mn during 35 years.
3	(Al-Amrah 2014)	K.S.A.	Cross sectional	40	WTS=20 NS=20	40	0	Between 28 and 65 years	Males daily smoke jurak or moassel WTS/ not / no cigarettes ,not alcoholic /no medications / middle class.
4	(Azab 2015)	Jordan	Cross-secional	112	WTS =66 NS=46	WTS=34 NS=23	WTS=32 NS=23	WTS=30.2 ±10.3 NS= 31.9 ± 11.1	Healthy WTS and age-matched healthy nonsmokers

5	(Eker 2016)	Turkey	Cross-sectional	60	WTS=30 NS=30	-----	-----	Average range 18 to 25 years	WTS smoked an average of 2 times per week and not smoke cigarettes. NS who had never smoked.
6	(Naderi 2017)	Iran	Cross-sectional	75	WTS=25 CS=25 NS=25	75 WTS=25 CS=25 NS=25	---------	Between the age 25 and 50 years old	Males with no systemic diseases ,not taking any medication ,not exposed to radiation in the last 6 months . WTS. participants who never smoked cigarettes or use less than 100 in whole life.
7	(Silveira 2017)	Brazil	Cross-sectional	80	WTS=40 NS=40	WTS=20(NAC=2/MAC=18) NS=20(NAC2/MAC=18)	WTS=20(NAC=5/MAC=15) NS=20(NAC=4/MAC=16)	WTS=22.55± 3.02 NS=20 ±3.1	WTS. paired by gender, age and alcoholic habits. exclusion of diseases /WTS. not ex cigarettes smokers or with smokeless tobacco habit.
8	(Amer 2018)	Egypt	Cross-sectional	64	WTS=16 CS=16 WTS+CS=16 NS=16	---------	--------	----------	Healthy/ no apparent, oral lesions;. exclusion systemic conditions/no other exposed to carcinogenic substance .
9	(Taghibakhsh 2019)	Iran	Cross sectional	72	WTS=36 NS=36	72	0	WTS=27.3± 5.9 NS=29.9± 6.1	no, alcohol and drug consumption/no systemic disease, head and neck radiotherapy,/no chemical agents.
10	(Prasad 2019)	UAE	Cross sectional	-400	WTS=100 CS=100	367	33	----------	----------

					WTS+CS=100 NS=100	WTS=89 CS=100 WTS+CS=90 NS=88			
11	(DehghanNezhad 2020)	Iran	Cross sectional	60	60 WTS=30 NS=30	60 WTS=30 NS=30	0	between the age 20 to 50 years old	Exclusion of participant less than 20 years old with/no systemic diseases / not alcohol consumers /not exposed to dental radiation with the last six months.
12	(Rajabi-Moghaddam 2020)	Iran	Cross-sectional	90	WTS=30 CS=30 NS=30	90	0	Between 18 and 25 WTS=24.7 ± 6.26 CS=23.83 ± 6.93 NS=22.6 ± 5.25	Male (age18 - 25)years/ no clinically visible lesion in their mouth/ no history of malignant or premalignant lesions or conditions.

P =participants /WTS=waterpipe tobacco smokers/CS=cigarettes smokers/NS=non-smokers/NAC=non-alcohol consumer /MAC=moderate alcohol consumer.

Table 5: Extracted data for the sample and exposure types and outcomes.

No	Citation		Analysis Method	WTS duration and consumption	CS duration and consumption	Cellular Biomarker	Molecular Biomarker	Outcome
1	(El-Setouhy 2008)	Exfoliated buccal mucosa	A modified Papanicola-ou method	Number of hagars/week≤ 28 hagars N=67 mn= 10.2 SD=3.9 >28 hagars N=59 mn=10.3 SD= 3.7 Duration of smoking	----------	MN	----------	mean TMN in WTS and NS 10.2 ± 3.9 vs. 4.1 ± 1.9, p <0.001 mean CMN in WTS vs NS 8.0 ± 3.2 vs. 3.7 ± 1.6, p <0.001

				≤ 14years mn= 10.6 SD=3.7 >14years mn=9.9 SD=4.1				
2	(Seifi 2013)	cytologic smear samples from buccal mucosa, lateral surface of the tongue, and floor of the mouth (right)	Papanicolaou staining technique	smoke once or twice/week for 20-80 mn for 35 years	CS group smoke 10-40 cigarettes/day for 6 to 15 years	-nuclear and cytoplasmic size, - (N/C) and (F/R) ratio, -percent of KHC -vacuolization of cytoplasm, -two or multilobed nuclei.		a. Increase in nuclear size, the N/C ratio, and F/R b. Statistical result of this study did not indicate as statistical differences among the groups.
3	(Al-Amrah 2014)	Exfoliated buccal cells and (blood xx)	Comet assay	The frequencies of smoking 1 to 4 times a day.	----------	-Tail moment -Tail length -% Tail DNA	--------	Waterpipe smoking is directly implicated due to the damage caused in vivo in buccal cells of smokers and the tail moment and tail length in buccal

						-Fragmented DNA.		cells of smokers were statistically higher than control.
4	(Azab 2015)	saliva, (urine, and serum xx)	ELISA	Approximately 27% initiated WTS before the age of 17 years. In addition, 59% of WTS users were daily smokers, 68% smoked less than 1 h per session, and approximately half of the subjects smoked 2 heads per session. Most of the waterpipe smokers owned at least 1 waterpipe.	----------	----------	(8-OHdG) assay.	a-Levels of 8-OHdG in WTS vs NS in the saliva (52,430 ± 2923 vs 48,430 ± 4189 pg/mL). b-8-OHdG is smilar in WTS and NS
5	(Eker 2016)	Peripheral blood/buccal smear	Fleugen stain	An average of 2 times /week	----------	Micronucleu-s and binucleus ratio	----------	a. Micronucleus ratio was 6.03±2.06 and 4.43±2.27 ($p<0.05$) in the WTS and NS, respectively, b. Binucleus ratios were 8.53±3.23 and 12.15±5.18, respectively ($p<0.05$).

6	(Naderi 2017)	exfoliated buccal mucosa cells	Feulgen-stained	WTS Particepants never smoked cigarettes or smoked about 100 cigarettes in entire life the average duration was 4 years.	The average of smoking duration is 20 years .	The number of PYC ,KHC,and KL in 1000 cells/subject	----------	a. Significant differences among the groups in terms of karyolysis and pyknosis. b. Cytotoxicity effect of cigarette smoking was not significantly correlated to time exposure (r = 0.099, P = 0.637). c. The cytotoxicity effect of waterpipe smoking was significantly correlated to time exposure (r = −370, P = 0.044).
7	(Silveira 2017)	Exofoliated buccal cells	buccal micronucleus cytome test.(BMCYt)	Non cigerettes smokers but alcohol consumers ,WTS smoke at least 1 h per day for 1–2 days per week for at least one year(not using any other tobacco habits)	----------	- PYC - KL - KR - CC - BN - BC - NBUD - DIFF - MN	----------	a. An increasing P < 0.05 in all waterpipe smoker's cell parameters, except DIFF (fold-decrease). Only CC showed no differences between groups.

8	(Amer 2018)	salivary sample and a mucosal biopsy	Biopsy (H&E techniques) Saliva(a human cytokeratin fragment Antigen 21-1 (CYFRA21-1) ELISA Kit	(at least 3 times a week) and for a period not <5 years.	(at least 10 cigarettes a day) for a period not <5 years.	----------	P53 AND CYFRA 21-1	a. Statistically significant difference in p53 expression was present between nonsmokers and the three smoker groups.
9	(Taghibakhsh 2019)	Exofoliated buccal cells	Papanicolaou technique.	Not clear	----------	- MN - KHC - KYL - BE - Repair index = (KHC+KYL)/(MN+BE)	---------	a. Statistical significant between hookah users and controls (P<0.001).
10	(Prasad 2019)	Exfoliated buccal cells	Feulgen	Not clear	Not clear	MN		a. Comparison of Micronuclei in Feulgen and Micronuclei in Acridine Orange group between four groups was significant.

			stain and Acridine Orange stain			frequency was checked in 1000 cells.		b. Order of mean micronuclei with Fulegen and acridine orange was WTS+CSe > WTS > CS > Control groups.
11	(DehghanNezhad 2020)	Exfoliated buccal cells	Feulgen-stained	at least once in a week. Time duration of Smoking/ year N mean±SD 0-200 11 118±33.4 201-400 15 260±48.1 401-600 4 555±52.5	----------	MN		a. The micronuclei count in WTS was significantly higher than NS (P=0). b. The difference between the number of WTS and micronuclei count was significantly different (P=0).

12	(Rajabi-Moghaddam 2020)	Exfoliated buccal cells	Papanicolaou technique	at least one time per week for at least 1 year. The mean duration of consumption of WTS 5.26 ± 2.74 years.	at least 5 cigarettes /day for at least 5 years The mean consumption of CS was 270.81 ± 127.23 packs of cigarettes per year.	frequency of MN	----------	a. The mean number ± standard deviation (SD) of MN in waterpipe smokers, cigarette smokers, and nonsmokers was 7.55 ± 5.530, 4.95 ± 5.633, and 2.00 ± 2.406, respectively. b. The mean number ± SD of cells with MN in waterpipe smokers, cigarette smokers, and nonsmokers was 6.20 ± 4.830, 3.50 ± 3.832, and 1.45 ± 1.701, respectively. c. Numbers of cells with MN differed significantly between waterpipe smokers and cigarette smokers ($p = 0.04$) and between waterpipe smokers and nonsmokers ($p < 0.001$).

Basal cells (BC) / binucleated cells (BN) /Broken egg (BE) / cigarettes smokers (cs) / Condensed chromatin (CC) / Differentiated cells (DIFF) / Karyolitic cells (KYL) / Karyolysis (KL)/ Karyorrhetic cells (KHC)/ Karryorrhxis (KR) / Mean number(mn) /Moderate alcohol consumer(MAC) / Micronuclei (MN)/ Non-alcohol consumer (Nac) / Non-smokers (NS)/ nuclear buds (NBUD) / nuclear-cytoplasmic (N/C)/ Participants(P) /Waterpipe tobacco smokers(WTS) /8-hydroxy deoxyguanosine (8-OHdG) assay/ Xx=will not be included in our review.

3.3 QUALITY ASSESSMENT OF THE STUDIES

The studies were assessed independently by two reviewers (authors DE and TR), and a third reviewer (FKD) acted as a mediator to resolve disagreements that could not be resolved by discussion. The quality assessment results have been reported in narrative form and as indicated in Tables (6), regardless of the methodologies' quality adopted in the studies.

In the risk of bias categories, the studies demonstrated different risks of bias, namely high, moderate, low, and very low risks. The overall evaluation of the risk of bias suggested that four studies had a high-risk Eker (2016); Amer (2018); Taghibakhsh (2019); Rajabi-Moghaddam (2020), five studies as moderate risk Seifi (2013); Al-Amrah (2014); Azab (2015); Naderi (2017); Silveira (2017), two studies as a low-risk Prasad (2019); DehghanNezhad (2020), and a final one scored as very low risk (El-setouhy 2008) illustrated in table (6).

Except for Seifi (2013) and Azab's (2015) cross-sectional studies, the remainder of the studies were poorly designed. Six reviewed studies omitted to describe their design, but they were considered cross-sectional based on their layout. These studies are: El-Setouhy (2008) Al-Amrah (2014), Eker (2016), Silveira (2017), Amer (2018), Prasad (2019). The following were considered case-control studies: Naderi (2017), DehghanNezhad (2020) and Rajabi-Moghaddam (2020). However, the presence of a comparison group did not change that it was a cross-sectional study. Finally, Taghibakhsh (2019) study was considered a historical cohort study, but that was not correct because it did not depict the fundamentals of the cohort study, such as differences in commonality.

All the evidence from the reviewed articles were marked by a minimum of three zones of high risk, and most by far more. At overall judgment of the 12 included studies , only two studies had a correct study design Seifi (2013) and Azab(2015). Also, some studies gave a complete description of the participants and specified their inclusion and exclusion criteria. However, four studies did not provide a complete description of the study settings Azab (2015); Eker (2016) Silveira (2017); Rajabi-Moghaddam (2020). According to the studies, all authors agreed that the high heterogeneity in this review lacked a standardized method to measure the exposure in the

WTS groups and CC groups; instead, included studies used self-reporting data to measure the exposure. For example, scores were allocated to responses with 'Yes' to measure WTS and CC by the self-reporting method. Self-reporting is a frequently used indicator in epidemiological studies for cigarette exposure (Etter and Perneger 2001). However, there is a concern that self-reporting methods are subjective and inaccurate. Also, there was a general agreement that the lack of a standardized method of recording the exposure may have compromised the validity of the results to some degree. Seven articles identified confounding factors, but only four were clear in their strategies when dealing with confounding factors.

Consequently, it was suggested that an adjustment for the potential confounding factors across all studies needed to be considered. These included gender, age, occupation, educational status and economic status. In this case, all authors agreed about the incomplete records of the study outcome inability to measure validity and reliability, and most of the articles failed to mention an expert or qualified person who conducted the outcomes measurement. Lastly, all studies had an accurate statistical analysis.

Table 6: Critical appraisal tool by JBI for cross sectional studies

Name of Author	Were the criteria for inclusion in the sample clearly define?	Were the study subjects and the setting described in detail?	Was the exposure measured in a valid and reliable way?	Were objectives, standard criteria used for measurement of the condition?	Were confounding factors identified?	Were strategies to deal with confounding factors stated?	Were the outcome measured in a valid and reliable way?	Was appropriate statistical analysis used?	Risk of bias suggested
(El-Setouhy 2008)	Unclear	Yes	Yes	Yes	Yes	Yes	Unclear	Yes	Very low
(Seifi 2013)	Yes	Yes	Yes	No	No	No	No	Yes	Moderate
(Al-Amrah 2014)	Yes	Yes	Unclear	Yes	Yes	No	Unclear	Yes	Moderate
(Azab 2015)	No	No	No	Yes	Yes	Yes	Unclear	Yes	Moderate
(Eker 2016)	No	No	No	Yes	No	No	Unclear	Yes	High
(Naderi 2017)	Yes	Yes	No	Yes	No	No	Unclear	Yes	Moderate
(Silveira 2017)	Yes	No	Yes	Yes	Yes	No	Unclear	Yes	Moderate
(Amer 2018)	Unclear	Unclear	No	Yes	No	No	Unclear	Yes	High
(Taghibakhsh 2019)	Yes	Yes	No	No	No	No	Unclear	Yes	High
(Prasad 2019)	No	Yes	No	Yes	Yes	Yes	Unclear	Yes	Low
(DehghanNezhad 2020)	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Yes	Low
(Rajabi-Moghaddam 2020)	Yes	No	No	No	Yes	No	Unclear	Yes	High

3.4 RESULT OF SYNTHESIS

This section will present and discuss the results of synthesized studies reviewed in this thesis. All of the outcomes of different articles are presented in detail in Table (5).

Primary outcomes

A) Molecular biomarkers

Most of the evaluated studies were cellular biomarkers except for three (n=3) that reported molecular biomarkers. In the study by Amer (2018), P53 and CYFRA21-1 were measured by buccal mucosal biopsy and saliva samples. Consequently, a higher expression of p53 in the three smoking groups was recorded. However, no statistically significant changes were found in the CYFRA 21-1 level among the four groups, whereas a statistically significant difference in p53 expression was present between nonsmokers and the three smoker groups. The study conducted by Azab (2015) focused on evaluating the levels of 8-OHdG in WTS vs NS in the saliva. The authors reported biomarker levels of 52,430 ± 2923 vs 48,430 ± 4189 pg/mL in the respective groups. From these findings, the author concluded that 8-OHdG did not correlate with WTS. Al-Amrah (2014) reported the mean number and the standard deviation in the tail moment and tail length as 186 ± 26 and 456 ± 71, respectively. The reported standard deviation was suggestive of damage to the buccal mucosa experienced by WTS because the standard deviation values were higher than the corresponding value for the NS group.

B) Cellular biomarkers

1/ Micronuclei

In Table (7), seven studies (n=7) reported the effect of WTS on the buccal mucosal MN. The effect of MN on buccal epithelial cells was expressed as the total number of MN in 1000 epithelial cells, total number or ratio of MN in 2000 epithelial cells, frequency, mean percentage, and cell

parameter. Different statistical analysis methods and laboratory techniques were used to evaluate the MN.

The study by Rajabi-Moghaddam (2020), compared the MN among the different groups of participants, namely WTS, CS and NS groups. The Prasad (2019) study evaluated the MN in four groups of participants: WTS, CS, NS and DS groups. Silveira (2017) analyzed 2000 cells by counting micronucleated cell parameters in WTS and a control group. Taghibakhsh (2019) showed that the mean percentage of MN in 500 cells increased in the WTS group compared to the NS group. Eker (2016) counted MN ratio in 2000 cells. The author recorded 6.03±2.06 and 4.43±2.27 MN in WTS and NS groups, respectively. Rajabi-Moghaddam (2020) and El-Setouhy (2008) compared the total mean number of MN per 1,000 cells in WTS and NS using Papanicolaou technique. Both studies reported that the mean total number of MN in WTS were higher than in non-smokers. Similarly, Rajabi-Moghaddam (2020) reported a lesser increase in the mean total number of MN in CS than in WTS.

2/Pyknosis

A two-fold increase in PYK parameter was observed in the WTS group compared to the NS group in Silveira's study (2017). Naderi (2017) showed that the mean number of PKY in the NS, WTS and CS groups was 0.17 ± 0.08, 2.44 ± 1.51, and 3.64 ± 3.13, respectively. These results indicate that the CS has more a damaging effect on human cells than the WTS.

3/Karyorrhexis

The percent of KHC was not statistically significantly different in the oral mucosae, tongue and floor of the mouth of WT users (p=0.8) and non-WT users (p=0.9) in the Seifi (2013) study. The study conducted by Naderi (2017) reported that the mean number of karyorrhexis in NS, CS, and WTS was 0.96 ± 0.15, 5.08 ± 3.32, and 4.76 ± 2.83 respectively, while the study conducted by

Silveira (2017) observed a significant increase ($P < 0.05$) in all waterpipe smoker's cell parameters. Taghibakhsh (2019) study revealed that the KHC scores in the hookah users and controls were 0.1±0.06 and 0.04±0.06.

4/ Karyolysis

Silveira (2017), reported a significant increase ($P < 0.05$) of KL in all WTS cell parameters, and Naderi (2017) reported the mean number of KL in 1000 cells/subject in nonsmokers, cigarette, and waterpipe smokers were 1.21 ± 0.19, 9.24 ± 5.81, and 4.24 ± 2.24 respectively.

Other biomarkers

Taghibakhsh (2019), examined the BE and the repair index = (KR+KL)/(MN+BE). The BE and repair index represent nuclear damage and evidence of carcinogenesis and cellular apoptosis. The BE score was higher in the WTS group than the NS group. As for the repair index, it was less in the WTS group than the NS group. Silveira (2017), detected two fold increase in all BN, BC, and nuclear buds cell parameters in the WTS group. However, only condensed chromatin (CC) decreased in DIFF of all groups. Seifi (2013) observed the highest increase in the nuclear size and the nuclear-cytoplasmic ratio was found in the CS group followed by WTS and NS groups, at the buccal mucosa, tongue, and floor of the mouth while a decrease in cytoplasm size in cigarette, waterpipe users and ordinary individuals, respectively. Summary of all the outcomes are presented in Table (8)

Secondary outcomes

We realized that cigarette or waterpipe smoking has a higher genotoxic effect on human oral cells or saliva in all the studies except Azab (2015), whose study was unable to establish a relationship between the biomarker OHdG and WTS participants. Also, Amer (2018) study failed to identify any change in CYFRA 21-1 among any groups. However, Naderi (2017) reported that the cytotoxicity effect of WTS was significantly associated with the time of exposure, unlike the CS, which has no association with time exposure and DehghanNezhad (2020) study, showed that the WTS cytotoxic effect was dose-dependant. Furthermore, El-Setouhy (2008) declared that WTs

had a higher genotoxic action because they increased the number of micronucleated cells in human oral tissue. Al-Amrah (2014) reported that the WTS caused more DNA damage represented by the tail moment and tail length in buccal cells of smokers than non-smokers. Eker (2016), showed that significant statistical differences occur in MN and binucleus ratio between the WTS and Ns groups suggesting that the WTS increases the genotoxic effect on the human oral cells. Silveira (2017), reported that waterpipe smoking causes more cellular damage, cell death and cell cycle disturbances putting users at high risk of cancer. Taghibakhsh (2019) study, showed that the repair index was lower and the percentages of all the examined cells were higher in the WTS group than no-smokers and concluded that WTS is responsible for more cellular damage. DehghanNezhad (2020), explained that the cytotoxic effect of WTS was dose-dependent and resulted in higher micronuclei counts in WTS users. This illustrated that the genotoxic effect caused by WTS was significantly higher in WTS users than in NS.

Seifi (2013), concluded that CS has a higher cytometric effect than WTS and NS. Also, in the study by Naderi (2017), the CS group recorded a higher rate of cellular death than WTS, represented by increased PYK and KL. Amer (2018) reported no statistically significant difference in P53 expression in either of the three smoker groups nor changed the level of CYFRA 21-1 in the three smokers group or the non-smoker group. Prasad (2019) showed that the

order of mean MN with Fulegen and acridine orange was higher in the Dual smokers (DS) group, followed by WTS, CS, and the Control groups. Finally, Rajabi-Moghaddam (2020) study, showed that the mean total number and the frequency of MN were higher in the WTS than in the CS group, while the study by Azab (2015), expressed that the level of OHdG was similar in WTS and NS.

Table 7: Micronuclei outcomes in included studies

NO	Citation	Sample Type	Groups	Analysis method	MN measurement
1	(El-Setouhy 2008)	Exfoliated buccal cells	WTS/ NS	A modified Papanicola-ou method	(TMN) was defined as the total number of MN per 1000 cells per subject. (CMN) was defined as the number of cells containing MN per 1000 cells per subject
2	(Silveira 2017)	Exfoliated buccal cells	WTS /NS	buccal micronucleus cytome test.(BMCYt)	2000 differentiated cells were analyzed counting micronucleated cells (MN) cell parameter
3	(Eker 2016)	Exfoliated buccal cells	WTS/ NS+ alcohol consume	Fleugen stain	Number of micronucleus in 2000 cells counted
4	(Taghibakhsh 2019	Exfoliated buccal cells	WTS/ NS	Papanicolaou technique	The mean percentage of MN(500 cells randomly)

5	*(Prasad 2019)*	Exfoliated buccal cells	WTS/ CS/W TS+CS /NS	Feulgen stain and Acridine Orange stain	*MN frequency was checked in 1000 cells*
6	(DehghanNezhad 2020)	Exfoliated buccal cells	WTS/ NS	Feulgen-stained	The number of micronuclei per 1000 cells
7	(Rajabi-Moghaddam 2020)	Exfoliated buccal cells	WTS/ CS/NS	Papanicolaou technique	1/the mean total number of MN per 1,000 cells 2/the mean frequency of micronucleated cells (cells with MN) per 1,000 cells.

Participants(P) /Waterpipe tobacco smokers(WTS)/Cigarettes smokers(CS)/Non-smokers(NS)/Micronuclei(MN)/Total number of micronuclei(TMN)/Cell of micronuclei(CMN)

Table 8: Summary of the outcomes

	citation	Group of exposure by order		
Micronuclei	(El-Setouhy 2008)		WTS > NS	
	(Silveira 2017)		WTS > NS	
	(Eker 2016)		WTS.> N.S.	
	(Taghibakhsh 2019		WTS > NS	
	(Prasad 2019)		DS >WTS >CS > NS	
	(DehghanNezhad 2020)		WTS > NS	
	(Rajabi-Moghaddam 2020		WTS> CS > NS	

Pyknosis	(Silveira 2017)		WTS > NS	
	(Naderi 2017)		CS > WTS> NS	
	(Seifi 2013)		WTS= NS	
Karyorrhexis	(Naderi 2017)		CS > WTS > NS	
	(Silveira 2017)		WTS > NS	
	(Taghibakhsh 2019)		WTS > NS	
Karyolysis	(Naderi 2017)		CS > WTS > NS	
	(Silveira 2017)		WTS > NS	
Broken eggs	(Taghibakhsh 2019)		WTS > NS	
Repair index	(Taghibakhsh 2019)		NS > WTS	

Binucleated cells, Basal cells and Nuclear buds	(Silveira 2017)		WTS> NS	
differentiated cells	(Silveira 2017)		NS>WTS	
condensed chromatin	(Silveira 2017)		WTS=NS	
Nuclear size, the Nuclear cytoplasmic ratio, and Feret ratio	(Seifi 2013)		CS> WTS >NS	
Cytoplasm Size	(Seifi 2013)		NS>WTS>CS	
Tail moment and Tail length	(Al-Amrah 2014)		WTS > NS	

p53	(Amer 2018)		WTS,CS,DS >NS WTS=CS	
CYFRA21-1	(Amer 2018)		Not significant	
8-OHdG	(Azab 2015)		Has no correlation with WTS	

Cigarettes smokers(CS)/Dual smokers of waterpipe and cigarette (DS) /Micronuclei(MN) / Nonsmokers(NS)/Waterpipe tobacco smokers(WTS)

3.5 Meta-analysis

Homogenous data were grouped to conduct a meta-analysis. Four studies counted the micronuclei in 1000 cells Prasad (2019); DehghanNezhad (2020); Rajabi-Moghaddam (2020); El-Setouhy(2008). Prasad's (2019) investigation was considered as two separate studies as the experiment was repeated twice with two different histological stains (Feulgen stain and Acridine Orange). Table (6) illustrates the MN frequency in 1000 cells in all included studies regardless of the laboratory technique. It is important to note that all the laboratory analyses were done under a light microscope. The dual smoking group, those using both WTS +CS, had the highest MN frequency followed by WTS, CS and NS. Apart from the sample size of the selected studies, the data homogeneity concerned the MN, with Prasad (2019) studies providing a comprehensive insight into the MN in 1000 cells. Of the selected studies, the mean numbers for the different categories of participants were highest for Dual smoking at 17.88. Also, the mean numbers and standard deviation of Prasad (2019) followed an increasing order for Dual smoking> CS> WTS> NS. Specifically, the order of magnitude for the standard deviation of NS and WTS was 7,6. This implies that a combination of Dual smoking has severe and adverse health effects on participants. Although different laboratory methods were used, the homogeneity of the statistical outcomes concerning the magnitude of the SD for WTS, CS and NS provided essential observations about the relationship between the study participants. Also, and more importantly, the statistical significance concerning the SD and mean numbers showed that the frequency of the MN cells is associated with the participants' smoking status. The overbearing implication of these results can be attributed to the health complications of WTS and CS, while the NS depicted a comparatively lower number of biomarkers. Similarly, both the Prasad (2019) and DehghanNezhad (2020) studies, using identical laboratory techniques (Feulgen stain), showed comparatively identical SD values for MN in the NS and WTS groups in the study of DehghanNezhad (2020) while in the study of Prasad (2019), the SD for CS, Dual smoking and WTS were identical hence coefficient of variation is similar. Meta-analysis was performed using Stata version 17 software, the forest plot display the frequency of micronuclei in 1000 cells in the different group, figure(2,3,4,5,6).

The five forest plots display the mean of micronuclei in 1000 cell, the overall mean difference was 5.73(95% CI: 2.41 to 9.o5) P<0.001, I^2 = 98.66% between the WTS group and NS , 1.05(95% CI: -0.29 to 2.39) *P*=0.18, I^2 = 39.15% between the WTS group and CS group , 6.24(95% CI: 1.88 to

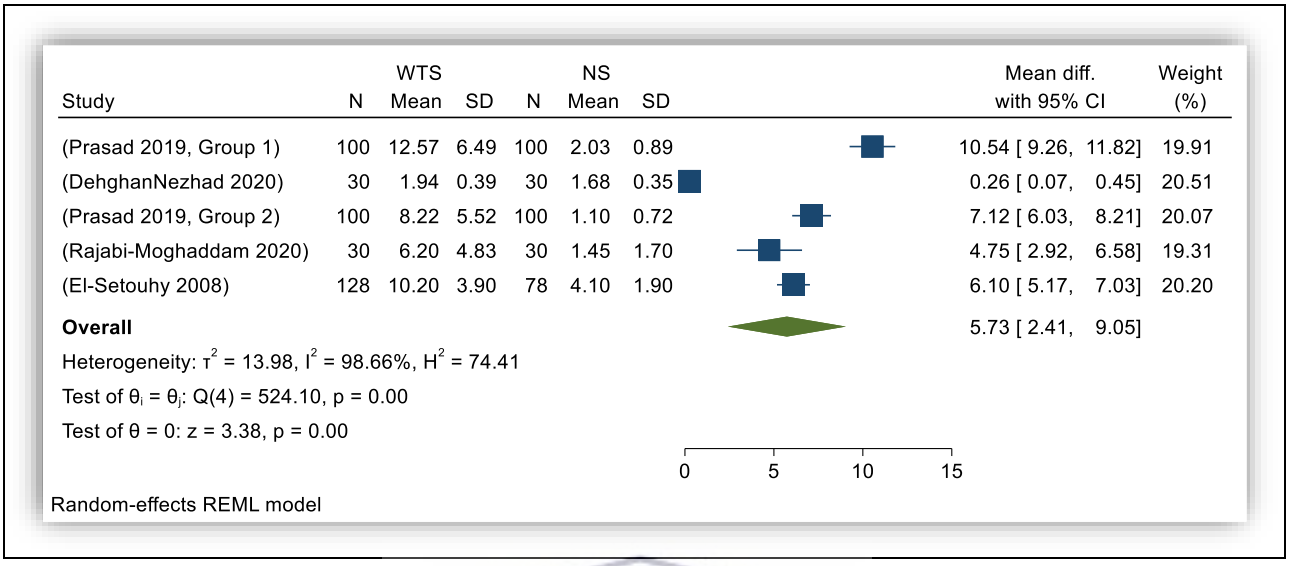
10.61) *P*=0.00, I^2 = 97.17% between the CS group and NS group , 13.89 (95% CI: 10.09 to 17.70) P=0.00, I^2 = 92.82% between the dual smoking group and NS group, 5.05 (95% CI: 3.75 to 6.35) P=0.73, I^2 = 0.00% between the Dual group and WTS.

Table 9: Frequency of Micronuclei per 1000 cell.

Citation	Lab Technique	Sam-ple size(NS)	NS (MN)	NS (SD)	Sam-ple size(WTS)	WTS (MN)	WTS (SD)	Sam-ple size(CS)	CS (MN)	CS (SD)	Sam-ple size(WTS+CS)	WTS+CS (MN)	WTS+CS (SD)
(Prasad 2019)	Feulgen stain	100	2.03	0.89	100	12.57	6.49	100	11.74	6.52	100	17.88	7.73
(DehghanNezhad 2020)	Feulgen stain	30	1.68	0.35	30	1.94	0.39	----	----------	---------	------	--------	--------
(Prasad 2019)	Acridine Orange	100	1.10	0.72	100	8.22	5.52	100	8.02	5.35	100	13.07	6.86
(Rajabi-Moghaddam 2020)	Papanicolaou technique	30	1.45	1.701	30	6.20	4.830	30	3.50	3.832	------	----------	---------
(El-Setouhy 2008)	A modified Papanicola-ou method	78	4.1	1.9	128	10.2	3.9	-------	----------	---------	-----	-------	---------

mean number (mn)/ Standard deviation (SD)/ Waterpipe tobacco smokers (WTS)/ Cigarettes smokers(CS) /Non-smoke

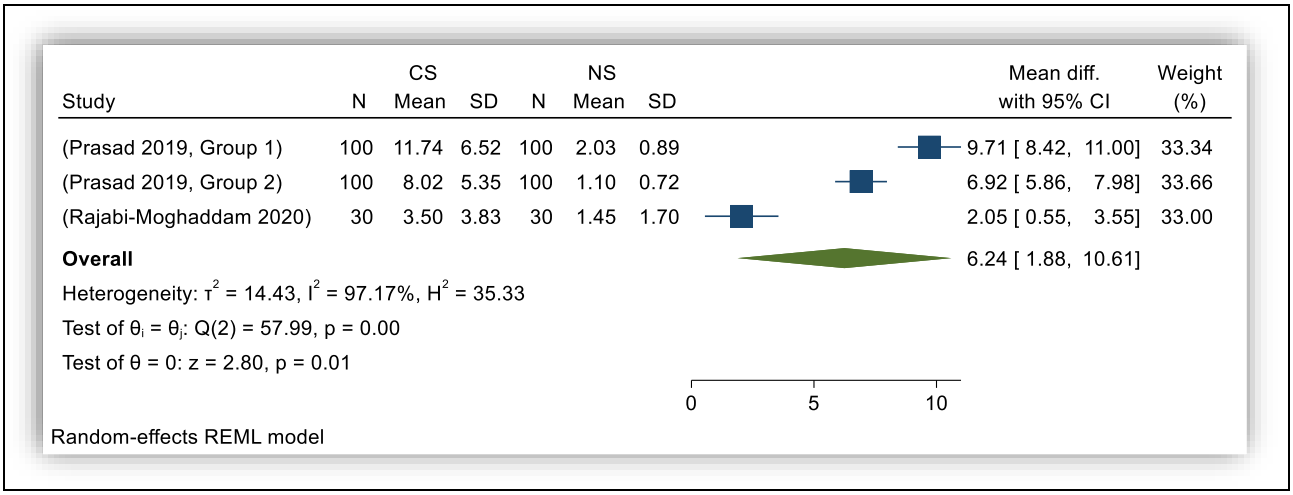
Figure 2: Mean MN/1000 cells for WTS vs NS



Meta-analysis was performed using STATA version 17 software, the forest plot presented the mean MN per 1000 cells was 5.73(95% CI: 2.41 to 9.o5) for WTS compared to NS. There was high heterogeneity as displayed by the I^2 of 98.66% and the p<0.001. We can say that the studies, El-Setouhy (2008), Prasad (2019) group 1, Prasad (2019) group 2, and Rajabi-Moghaddem (2020) were heterogeneous.

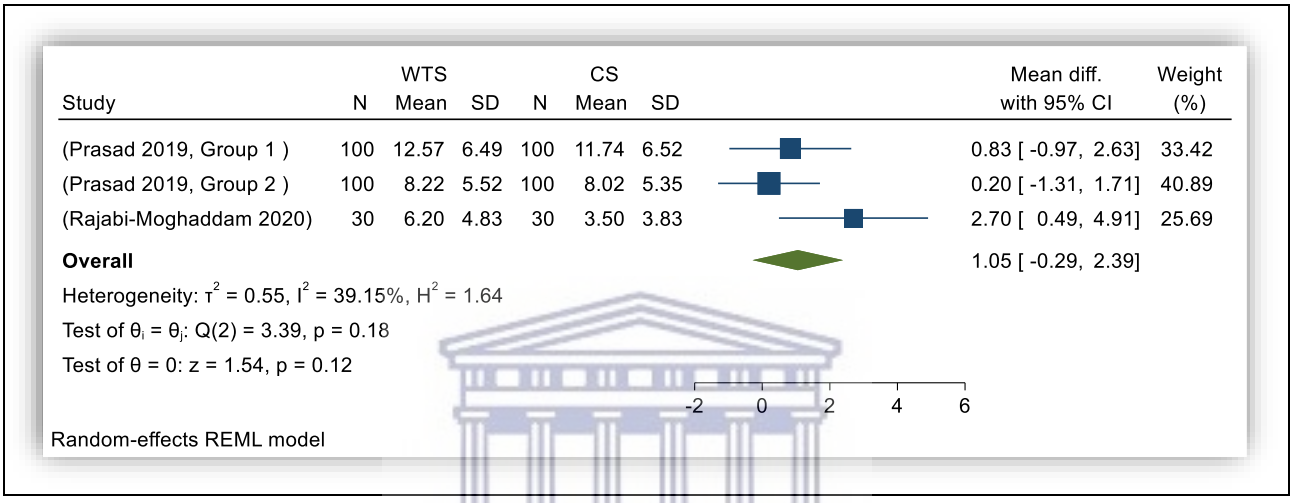
*Prasad (2019) group 1 done with Feulgen stain , Prasad (2019) group 2 done with Acridine Orange.

Figure 3: Mean MN/1000 cells for CS vs NS



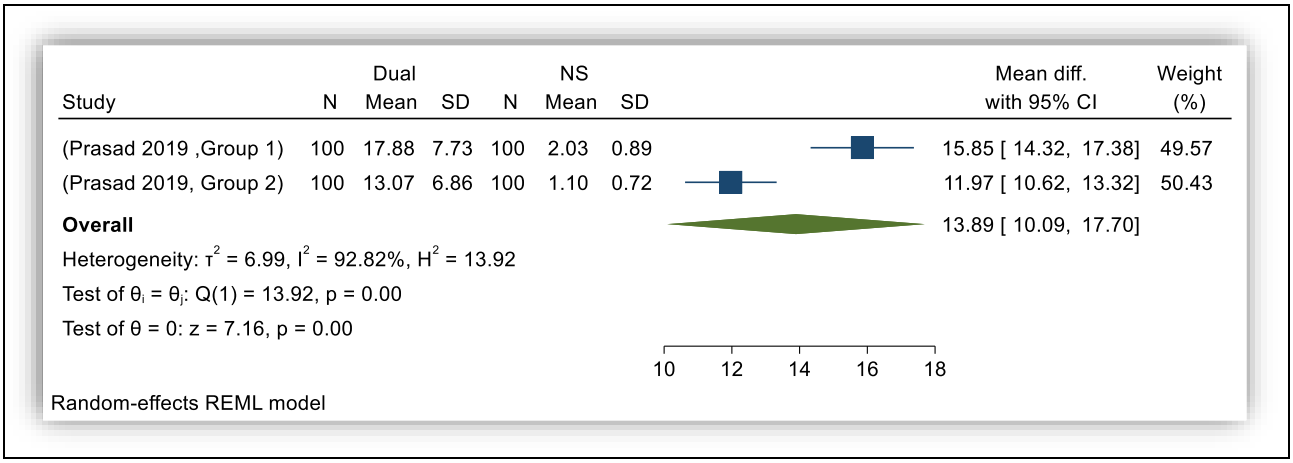
The forest plot presented the mean MN per 1000 cells was 6.24(95% CI: 1.88 to 10.61) for CS compared to NS. There was high heterogeneity as displayed by the I^2 of 97.17% and the p=0.00. We can say that the studies, Prasad(2019) group 1, Prasad(2019) group 2 and Rajabi-Moghaddem(2020) were heterogeneous.

Figure 4: Mean MN/1000 cells for WTS vs CS



The forest plot presented the mean MN per 1000 cells was 1.05(95% CI: -0.29 to 2.39) for WTS compared to CS. There was low heterogeneity as displayed by the I^2 of 39.15% and the p=0.18. We can say that the studies, Prasad (2019) group 1, Prasad (2019) group 2 and Rajabi-Moghaddem (2020) were homogenous.

Figure 5: Mean MN/1000 cells for Dual smoking vs NS



The forest plot presented the mean MN per 1000 cells was 13.89 (95% CI: 10.09 to 17.70) for Dual smoking compared to NS. There was high heterogeneity as displayed by the I^2 of 92.82% and the p<0.001. We can safely say that the studies, Prasad (2019) group 1 and Prasad(2019) group 2 were heterogeneous.

Figure 6: Mean MN/1000 cells for Dual smoking vs WTS

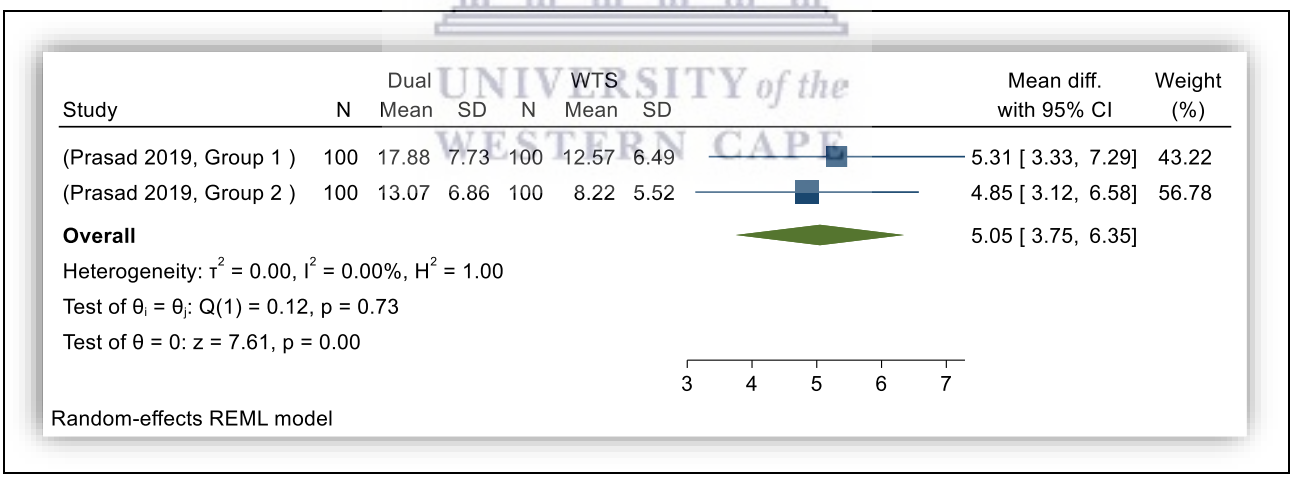


Figure (6) the forest plot presented the mean MN per 1000 cells was 5.05 (95% CI: 3.75 to 6.35) for Dual smoking compared to WTS. There was no heterogeneity as displayed by the I^2 of 0% and the p=0.73. We can safely say that the studies, Prasad (2019) group 1 and Prasad (2019) group 2 were homogenous.

3.6 Subgroup analysis and investigation of heterogeneity

We were dedicated to recording results from original studies by gender, age, type of exposure and outcomes where available. We reported most of our data results narratively since quantitative pooling of data across studies was restricted.

3.7 Sensitivity analysis

Due to the small number of included studies in the meta-analysis, a sensitivity analysis could not be conducted.

3.8 Reporting bias assessment

As mentioned in our protocol, if there were more than 10 included studies in the meta-analysis (Lam et al., 2020), publication bias would be demonstrated in a funnel plot, but our meta-analysis included only four studies.

4. Discussion

The review included twelve studies (n=12) with 1379 participants (541 nonsmokers, 511 waterpipe tobacco smokers, 211 cigarettes smokers, 116 dual smokers(cigarette and waterpipe tobacco smokers). The gender distribution was 1127 males and 128 females. Two studies with 124 participants did not indicate the gender of the participants Aker (2016); Amer (2018). Mainly adolescents and adults were included in the studies reviewed. However, the studies by Amer (2018) and Prasad (2019) did not provide explicit information about the participants' age. For instance, there were 11 studies conducted in the Middle Eastern countries, namely: Iran, Jordan, Kingdom of Saudi Arabia(KSA), the United Arab Emirates (UAE), Turkey and Egypt. Also, only one study was conducted in South America (Brazil). The heterogeneity of the reviewed studies was significant because participants' demographical characteristics, including age and gender, and other information, including the degree of exposure, sample type, laboratory technique and the method of study, varied considerably.

The cellular and molecular biomarkers analyzed resulted in interesting findings. For instance, the biomarkers showed varying statistical significance among different studies, resulting in different outcomes of WTS and Cigarette consumption. Exposure to WTS and CS can cause extensive damage to cells, genotoxicity and overall DNA disruption and can pose prolonged and complicated health risks to this group of individuals. In addition, WTS and CS cause the release of chemicals (which was unreported or discussed in the studies reviewed) from consumed products, especially over extended periods.

Simple, non-invasive techniques, including exfoliative cytology, can be used to assess the nuclear changes of buccal mucosal cells. This method also allows one to evaluate DNA adduct formation resulting from exposure to tobacco smoke (Stich 1984). Furthermore, the procedure can identify

nuclear changes in buccal mucosal cells, including MN, a biomarker of tissue carcinogenicity and genetic alteration (Palaskar et al., 2010). In addition, damage to buccal mucosal cells and nuclear changes such as KR, KL, PYK , BE, and the repair index are biomarkers that can also be detected by exfoliative cytology (Farhadi 2017).

Biomarkers serve different purposes depending on the objective of the study. Assays can be categorized into four types, and their use is determined by the extent to which a potentially harmful product must be evaluated. According to the Institute of Medicine, Committee biomarkers are classified into four groups: external exposure measurements, internal exposure biomarker, biologically effective dose estimation biomarkers, and potential harm biomarkers (Perera 1987; Bondurant et al., 2001). The cells investigated using these biomarkers of potential harm could act as surrogate markers and be used to determine actual harm, pre-clinical and clinical studies or diseases. In this study, the biomarkers of potential harm were used because we can detect the carcinogenic effect of toxicant exposure on human cells.

Micronuclei are found in the nuclei of cells and are identical to but smaller (about a third of) than primary nuclei. MN cells are round-to-oval, have well-defined margins and have identical colours (Kamboj et al, 2007). Micronucleus assay, developed by Schmid (1975), is routinely used to assess tissue carcinogenicity and genetic alterations. Other less-used nuclear changes such as karyorrhexis, karyolysis, and pyknosis are essential cellular health and disease indicators. During karyorrhexis, the nucleus of a dying cell becomes fragmented, and the chromatin is distributed unevenly throughout the nuclear cytoplasm. This gives the nucleus a dark appearance, and it disappears over time. Karyolysis is the complete dissolution of the nuclear chromatin and offers insights into the degree of cell death (Kumar *et al.*, 2010). Additionally, changes in the nucleus can occur due to nuclear damage with the broken egg's nucleus that is characteristically known as the worn nucleus (Tolbert *et al*., 1992).

Several reviews have focused on understanding the indicators of cell damage, including the frequency of MN changes in cells of smokers. However, very few have studied other nuclear changes in the same group of individuals (Kamboj *et al*., 2007; Farhadi 2017). In the present review, seven studies demonstrated the effect of WTS on MN of buccal mucosal cells. Three

studies (n=3) reported on pyknotic cells and karyorrhexis, two studies (n=2) reported on karyolysis, and only one study focused on other nuclear changes. In the current investigation, all the nuclear change indices were higher in WTS than in NS. Except for differentiated cells, the size of the cytoplasm was more prominent, and the repair index was higher in NS participants, indicating normal body hemostasis. The amount of condensed chromatin was the same in both WTS and NS groups. CYFRA21-1 and 8-OHdg did not show a correlation with WTS, and this implies that OHdG or CYFRA21-1 are not promising biomarkers for WTS (see Table 8).

Four studies compared WTS and CS Seifi (2013); Naderi (2017); Prasad (2019); Rajabi-Moghaddam (2020). Only two studies focused on nuclear changes Prasad (2019); Rajabi-Moghaddam (2020), and the authors reported that WTS caused extensive nuclear variations compared to CS. Also, WTS+CS, i.e. dual smoking groups, had the largest nuclear change index compared to other groups. Conversely, two Seifi (2013); Naderi(2017) studies found that the CS caused more nuclear changes than WTS. The disparity in study outcomes regarding the extent of nuclear change caused by waterpipe and cigarette smoking is noteworthy and suggests that additional underlying factors (confounders) may contribute to these changes. These results may refute the widely held misconception that passing tobacco smoke through water reduces its harmful effect. The findings of this review indicate that MN, KR, KL, PYK, and BE may be useful biomarkers to detect specific cellular nuclear changes in the buccal cells of tobacco smokers, but there is insufficient evidence to support their biological usefulness in WTS. Cellular damage caused by WTS and CS and the selectivity of cells affected by MN implies that continuous exposure and dosage determine the degree and severity of cell damage. Furthermore, the extent and severity of cell damage could be explained by the sensitivity of the tissues of the buccal cavity.

Ostling and Johanson (1984) developed the commet assay to study the passage of DNA fragments out of nuclei. This sensitive technique evaluates the toxicological genetic alterations in vitro and vivo. After cell injury, fragmented DNA passes from the nucleus to form a DNA strain called a comet tail that is measured by analyzing a “commet” to calculate the tail moments. Al-Amrah (2014) and Lu *et al.* (2017) used the comet assay to determine the mean number and the standard

deviation of the tail moment and tail length. In their investigations, the standard deviation reflected the buccal mucosal damage inflicted by WTS because this value was higher than the corresponding value for the NS group.

P53, the guardian of the genome, is a tumour suppressor gene that inhibits tumorigenesis, and its inactivation can result in cancer (Chen et al., 2021). A study conducted by Almutairi *et al*., (2021) showed an increase in all clinical parameters in smokers that these individuals are at a high risk of developing tobacco-related diseases due to the higher effector role of P53. Overexpression of the P53 can also indicate cellular alterations or cell cycle dysregulation. In our review, one study reported a higher expression of p53 in the three smoking groups (WTS, CS and dual smokers) compared to the non-smoking group, but the study could not report any statistically significant difference between the WTS and CS.

To the author's knowledge, this is the first systematic review that evaluated the cellular and molecular changes in WTS and compared them with CS. In this review, the high heterogeneity of the results may have resulted from the different methods of exposure measurements in the included studies. In essence, the lack of a standardized method or protocol for measuring participants' exposure to waterpipe and cigarette smoking. Another reason for the heterogeneity may have been the lack of expert or qualified personnel to measure the outcomes, leading to doubts about the recorded results. For instance, most of the studies evaluated the nuclear changes in the cells. The evaluation techniques may have been influenced by cost, accessibility, and availability of resources. It is noteworthy to mention that none of the included studies reported data on the DNA adduct or oxidative stress. These biomarkers may have been excluded from the investigations due to the complexity of laboratory technique, cost implication, and lack of expertise. Several studies have focused on biomarkers of tobacco exposure, mainly cigarettes, however, with relatively few studies involving WTS having been published.

The strength of this review is that all the steps of this research, data verification and the quality control measures were conducted independently by the two principal authors. Moreover, the steps were documented and recorded from the initiation of the protocol until the analysis of the results. Also, this research was conducted in strict accordance with PRISMA checklist that was used as a guideline. In addition, this study adopted the use of a standardized tool for quality assessment and meta-analysis.

The restriction and limitation of our review include that the search was restricted to Arabic and English language. Furthermore, the sources used to access scholarly resources was limited to four electronic databases of peer-reviewed journals. Furthermore, the data was limited to human in-vivo studies and excluded in-vitro or other experimental studies conducted on animals. Also, the included studies mainly included male participants, with relatively few studies reporting outcomes in females. This may have resulted from external factors such as cultural and religious inclination as in studies in the Middle East and mainly on WTS.

5. Conclusion

The use of WTS and CS compromises human health, especially the human oral cavity. The effectiveness of using appropriate biomarkers is a vital component in the diagnosis of disease. The present study highlighted the impact of waterpipe smoking and cigarette smoking on active tobacco users' oral mucosal cells and saliva. The changes observed in the mouth may be a reflection of those encountered in other tissues of the body. For instance, although biomarkers are used to analyze nuclear damage, the reported outcomes and comparison of studies did not indicate which consumed product, WTS, or CS as a more significant carcinogenic effect. Smoking has a cultural undertone, especially in the Middle East. However, the reviewed studies have shown the far-reaching impact of smoking if uncontrolled or unabated. Effective diagnosis and mitigating health protocols have become an urgent measure to ensure that the health and immunity of smokers are not compromised. The various studies reviewed in the study could serve as a tool to develop robust and effective interventions among smokers of WT and CS. There is a need for further research that explores oxidative stress, nuclear changes and DNA adduct biomarkers in WTS.

Protocol registration

The review was conducted according to the requirements contained within the PRISMA-P checklist for systematic review and meta-analysis. This protocol was registered with the PROSPERO registry of the University of York. The registration number is CRD42020209697 and is based on the preferred reporting items for systematic reviews and meta-analyses protocols (PRISMA-P) statement guidelines. Our research was also registered with the BMREC at the University of the Western Cape Registration number: BM20/9/4.

Contributors: The original thesis was drafted by (DE), read and revised by (FKD) and (TR) and the final manuscripts edited by (FKD) and (TR). All the authors agreed to the research methods, search strategy, data selection, data extraction, and publication. This review is to contribute towards a degree award for author (DE).

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7. Appendixes

PRISMA 2020 Checklist (Appendix 1)

Section and Topic	Item #	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	Page 1
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	Page 7
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	P 9
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	P15 (line4)
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	P16 line 9)
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	P17 (line3)
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	P17 (line6)
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	P19 (line12)
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	P20 (line 1)
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	P20 (line 7)
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	P20 (line 5)
Study risk of bias	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each	P20 (line

Section and Topic	Item #	Checklist item	Location where item is reported
assessment		study and whether they worked independently, and if applicable, details of automation tools used in the process.	18)
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	P21(line 14)
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	P21 (line 3)
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	P21(line 13)
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	P21 (line 7)
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	P21 (line11)
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	P21 (line18)
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	P21(line 18)
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	P21(line 26)
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	P22(line 1)
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	P22(line 12)
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	P22(line29)
Study characteristics	17	Cite each included study and present its characteristics.	P24(line1)
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	P42(line 1)
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	P37
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	P45
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	P55
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	----------
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	P61
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	P 61
Certainty of	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	----------

Section and Topic	Item #	Checklist item	Location where item is reported
evidence			
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	P 61
	23b	Discuss any limitations of the evidence included in the review.	P65
	23c	Discuss any limitations of the review processes used.	P65
	23d	Discuss implications of the results for practice, policy, and future research.	P66
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	P66
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	------
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	P66
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	P66
Competing interests	26	Declare any competing interests of review authors.	P66
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	----------

From: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71. doi: 10.1136/bmj.n71

For more information, visit: http://www.prisma-statement.org/

Study Eligibility Form (appendix 2)

Reviewer ID: ---------- Date of reviewing: ----------

Study ID: ----------

Eligible for inclusion (tick appropriate box)

	Yes	No	Unclear
Type of study 1/Case control 2/Cohort 3/cross-sectional			
Type of participants 1/adult 2/adolescence			
Exposure 1/WTS effect on the oral mucosa 2/WTS effect on the saliva			
Out come 1/cellular biomarker 2/molecular biomarker			

Included: ☐ **excluded**: ☐ **not clear**: ☐

Other reasons for exclusion/comments:----------

(appendix 3)

THE JOANNA BRIGGS INSTITUTE

JBI Critical Appraisal Checklist for Analytical Cross Sectional Studies

Reviewer..... Date.....

Author..... Year_____ Record Number_____

	Yes	No	Unclear	Not applicable
1. Were the criteria for inclusion in the sample clearlydefined?	☐	☐	☐	☐
2. Were the study subjects and the setting described indetail?	☐	☐	☐	☐
3. Was the exposure measured in a valid and reliableway?	☐	☐	☐	☐
4. Were objective, standard criteria used formeasurement of the condition?	☐	☐	☐	☐
5. Were confounding factors identified?	☐	☐	☐	☐
6. Were strategies to deal with confounding factorsstated?	☐	☐	☐	☐
7. Were the outcomes measured in a valid and reliableway?	☐	☐	☐	☐
8. Was appropriate statistical analysis used?	☐	☐	☐	☐

Overall appraisal: Include ☐ Exclude ☐ Seek further info ☐

Comments (Including reason for exclusion)